Candida albicans and host interaction: the many faces of the *Candida* Pra1 protein

Dissertation

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1. Abbreviations

AfCalAp	*Aspergillus fumigatus* extracellular thaumatin domain protein
AFAlp	*Aspergillus fumigatus* extracellular alkaline proteinase
AfD	*Aspergillus fumigatus* diffusible product
aHUS	atypical hemolytic-uremic syndrome
Als	Agglutinin-like sequence
AMD	Age related macular degeneration
AP	Alternative pathway
APC	Antigen presenting cell
Bac	β-protein
C1-INH	C1 inhibitor
C4BP	C4b-binding protein
ca.	circa
CFHRs	Complement Factor H related proteins
CHIPS	Chemotaxis inhibitory protein of *Staphylococcus aureus*
CR1	Complement receptor 1
CR2	Complement receptor 2
CR3	Complement receptor 3
CR4	Complement receptor 4
CP	Classical pathway
CRASPs	Complement regulator acquiring surface proteins
CRP	C-reactive protein
CCP	Complement control protein
CTL	Cytotoxic T lymphocyte
DAF	Decay accelerating factor
DNA	Deoxyribonucleic acid
DiO	3, 3'-dioctadecyloxacarbocyanine perchlorate
EACAM1	Carcinoembryonic antigen-related cellular adhesion molecule 1
Ecb	Extracellular complement-binding protein
ECM	Extracellular matrix
EED1	*Epithelial escape and dissemination 1*
Efb	Extracellular fibrinogen-binding protein
Ehp	Efb homologous protein
ELISA	Enzyme-linked immunosorbent assay
ΔFactor B-HS	Factor B depleted human serum
ΔFactor H-HS	Factor H depleted human serum
FB	Factor B
Fba	Fibrinogen binding protein
FD	Factor D
FHA	Filamentous hemagglutinin
fHbp	factor H-binding protein
FHL-1	Factor H like protein 1
FP	Factor P
PKH1	*Forkhead transcription factor 1*
gC	Glycoprotein C
GFP	Green fluorescence protein
GNA 1870	Genome-derived neisserial antigen 1870
gpA	Glycoprotein A
Gpm1	Phosphoglycerate mutase 1

HaCaT	Human adult low calcium temperature keratinocyte
HcpA	Human complement regulator and plasminogen binding protein A
Hgc1	Hypha-specific G1 cyclin-related protein 1
Hic	Factor H-binding inhibitor of complement
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
Hsf	*Haemophilus* surface fibril
HUVEC	human umbilical vein endothelial cell
Hwp1	Hyphal cell wall protein 1
HYR1	Hyphally regulated cell wall protein 1
iC3b	inactive C3b
IFN-γ	Interferon gamma
Int1	Integrin-like protein 1
kDa	kilodalton
LcrV	Virulence V-antigen
Len	Leptospiral endostatin-like protein
LfhA	Leptospira factor H-binding protein A
Lip	Lipase
LP	Lectin pathway
LSM	Laser scanning microscopy
MAb	Monoclonal antibody
MASP	Mannose binding lectin associated protease
MBL	Mannose binding lectin
MCP	Membrane cofactor protein
MFI	Median fluorescence intensity
mg	milligram
MHC	Major histocompatibility complex
µg	micro gram
µl	micro liter
ml	milli liter
MPGN	Membranoproliferative glomerulonephritis
NHS	Normal human serum
NK cell	Natural killer cell
NO	Nitric oxide
NS1	Nonstructural protein 1
Omp	Outer membrane protein
OPA protein	Opacity-associated protein
OPD	o-phenylenediamine dihydrochloride
PaAP	Alkalinprotease from *Pseudomonas aeruginosa*
PaE	Elastase from *Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
PspC	Pneumococcal surface protein C
Phr1	pH regulated protein 1
Phr2	pH regulated protein 2
Plb	Phospholipase B
PMNs	Polymorphonuclear leukocytes
Por1	Porin protein 1
Pra1	pH-regulated antigen 1
PrtH	*Porphyromonas spp.* protease
ROS	Reactive oxygen species
RT	Room temperature

Saps	Secreted aspartic proteinases
Sbi	Staphylococcal binder of immunoglobulins
SCIN	Staphylococcal complement inhibitor
Scl	Streptococcal collagen-like protein
ScpB	*Streptococcal C5a peptidase* (ScpB) of Group B *streptococci*
SCR	Short consensus repeat domain
SIC	Streptococcal inhibitor of complement
sTCC	soluble TCC
Stx2	Shiga toxin 2
T_C cell	T cytotoxic cell
TCC	Terminal complement complex
TCR	T cell receptor
TEF1	*Transcription elongation factor 1*
T_H cell	T helper cell
TLRs	Toll like receptors
TP	Terminal pathway
tPA	Tissue plasminogen activator
Tuf	Transcription elongation factor
uPA	urokinase-type plasminogen activator
UspA	Ubiquitous surface protein A
VacA	Vacuolating cytotoxin A
YopH	*Yersinia* outer protein H

2. Introduction

Hosts live in a close association with a great variety of infectious microbes, i.e. fungi, bacteria, viruses, protozoa and multi-cellular parasites. Therefore, host and microbe interaction happens every second within our lives. This interaction is complex and dynamic, and plays a key role for pathogenesis. The outcome of such an interaction may range from elimination of microbes, to infections caused by microbial pathogens [1] (**Figure 1**). Upon the course of infection, hosts utilize both physical barriers and an efficient immune defense system which includes the innate immunity and the adaptive immunity for protection. However, pathogens have evolved sophisticated infection strategies to cross tissue barriers, control and evade host immune attack. Thus, there is a balance between protective immunity and microbial infection [2]. At the end, who will succeed depends on the ability of the host to combat microbial infection and the ability of microbial pathogens to evade host immune defenses. How the host immune system is activated and regulated to eliminate any invading microbe and maintain cellular homeostasis, and how pathogens establish an infection will be explained in the following.

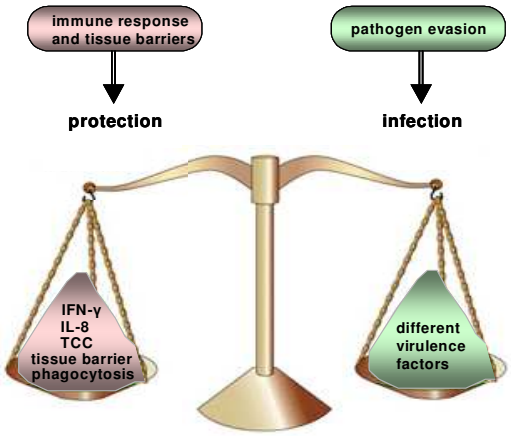

Figure 1. Balance of the protective immunity and microbial infection. Hosts utilize both tissue barriers and efficient immune responses, e.g. inflammation, phagocytosis, killing, complement mediated cell lysis and adaptive immunity for protection. Pathogens evolved different virulence factors and sophisticated evasion strategies for infection. The graphic was modulated from *Denise M. Monack, et al. Nature Reviews Microbiology. Sep. 2004.*

2.1 Overview of the host immune responses

The physiological function of the host immune system is to defense against infectious microbes. Broadly speaking, the protective host immune responses can be classified into two major categories: innate immunity and adaptive immunity. Both types are further divided into humoral and cellular immune responses, which are mediated by different proteins or cell types (**Figure 2**).

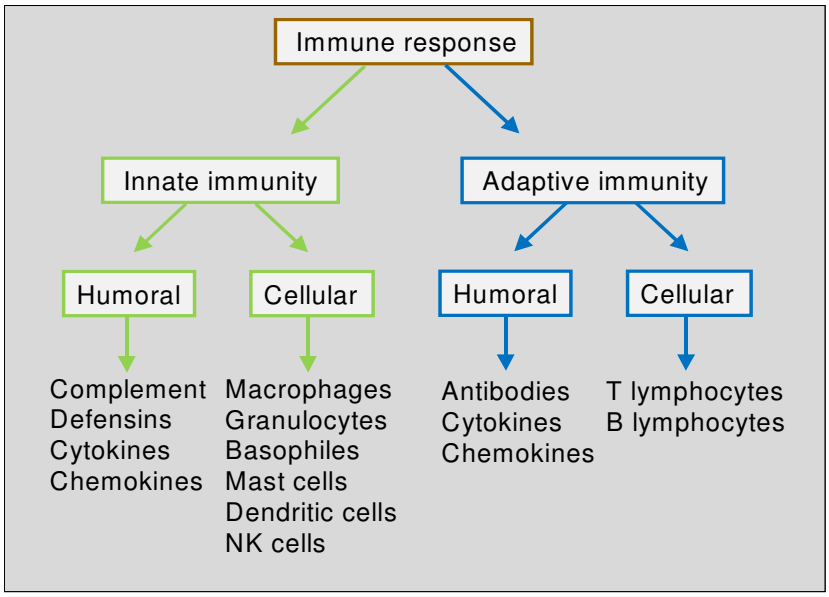

Figure 2. Classification of host immune responses and corresponding components

The innate immune system provides an early defense line that blocks the entry of the microbes, limits the growth of many infectious microbes that invade host tissues, and eliminates any harmful host cell [3,4]. The first interaction sites between individual microbial pathogens and their hosts are mainly the skin, gastrointestinal and respiratory tracts. These contact sites are lined by continuous epithelial cells that serve as barriers to prevent the entry of microbes from the external environment. If successfully crossing these epithelial barriers, microbial pathogens are immediately attacked by the next step of innate immune response, which is mainly mediated by complement system, neutrophils, macrophages and other leukocytes [5]. During this process, complement is fully activated and promotes destruction of the microbes and inflammation. In addition to complement activation, local resident phagocytes release cytokines and chemokines. These released immune effector molecules together with complement based C5a and C3a function as the "messenger molecules" to recruit neutrophils and macrophages to sites of infection. These activated leukocytes phagocytose and kill microbes, further release cytokines to recruit and activate more host leukocytes to infection sites. All these fast responses happen within seconds after infection until hours in most cases and efficiently control and eradicate many infectious microbes. In contrast to the fast innate immunity, adaptive immunity starts to function at the later stage after days of the infection (**Figure 3**). The adaptive immunity, which is specific, can distinguish between different, even close related microbes and molecules. The adaptive immunity utilize three main strategies to combat microbes: (i) secretion of antigen specific antibodies that bind to microbial antigens;

(ii) phagocytosis and killing of ingested microbes; and (iii) direct destruction of the microbe-infected host cells [6].

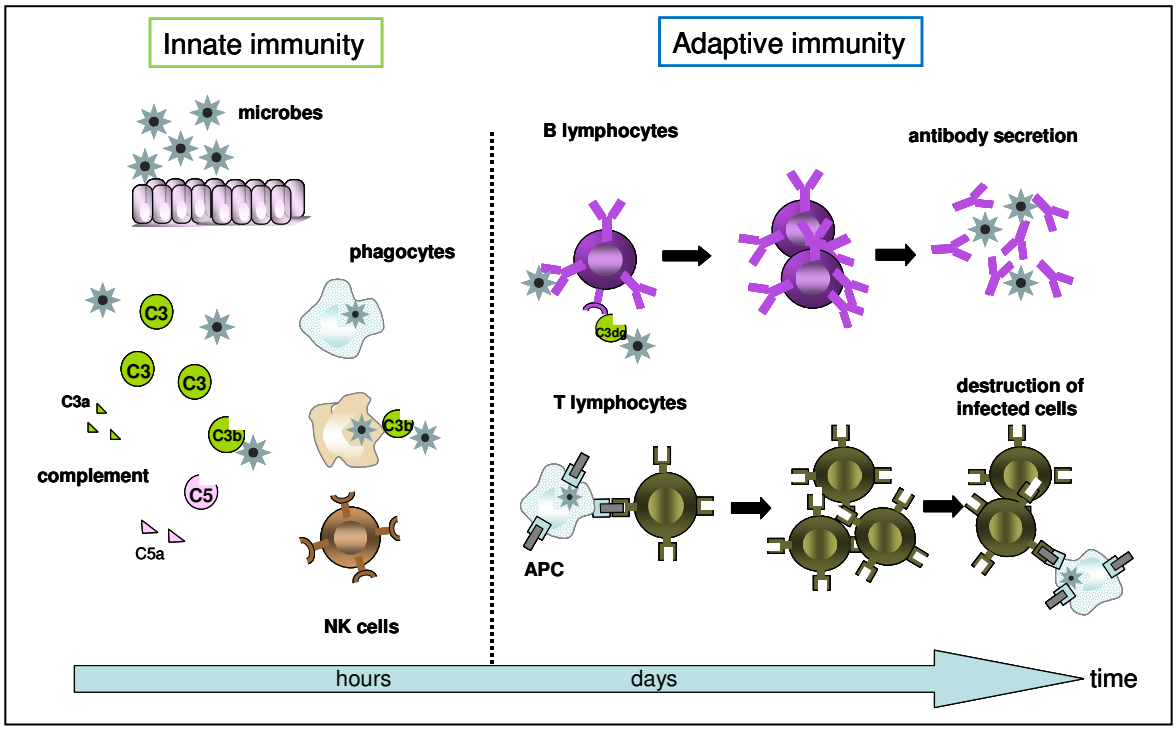

Figure 3. Overview of host innate and adaptive immune responses and corresponding cells. The innate immunity is composed of epithelial barriers, complement and immune cells (such as macrophages, neutrophils, dentritic cells and NK cells). The innate immunity provides fast and initial defense against infection. The adaptive immunity is initiated after days of infection, which developed by recognition, activation and effect function. Modified from the *"Cellular and molecular immunology by Abul K. Abbas, et al. 2009 (the 6th edition)"*.

2.2 Innate immunity

The innate immunity is the early defense line against infections agents. It is activated by the structures that are characteristic of microbes, but not available on mammalian cells. These conserved structures are called *pathogen associated molecular patterns* (PAMPs), such as lipopolysaccharides, teichoic acids and mannose-rich oligosaccharides. The receptors that bind PAMPs are called *pattern recognition receptors* (PRRs), including *toll like receptors* (TLRs), pentraxins, dectin-1 and ficolins. The principle components of the innate immunity are: (i) physical and chemical barriers, such as epithelia and anti-microbial substances produced at epithelial surfaces; (ii) the complement system as well as other inflammation mediators in the serum; (iii) macrophages, granulocytes (such as neutrophils, eosinophils and basophiles), mast cells, dendritic cells and natural killer cells (NK cells); and (iv) cytokines and chemokines that regulate and coordinate many activities of host innate immune responses [7]. Upon infection,

innate immunity uses two major effective systems to recognize and eliminate foreign and dangerous cells: the complement system and the cellular immune response.

2.2.1 Complement system

The term "complement" was originally applied by Paul Ehrlich to describe the activity of lysis of bacteria in an antibody containing serum. Later this lytic activity was further identified as a heat-labile serum component mainly by Bordet [8]. Complement contains more than 30 soluble and membrane bound proteins. Complement forms the first defense line of the innate immunity, and is distributed in plasma, body fluid, extra-cellular matrices, and also on the host cell surfaces [9,10]. Complement can be activated by three different pathways within seconds upon infections: (i) the alternative pathway, (ii) the lectin pathway; and (iii) the classical pathway. All three pathways end with a terminal pathway cascade (**Figure 4**). Complement activation leads to the rapid destruction of a wide range of invading microbes by direct lysis or promotion of phagocytosis and inflammation. The complement based biological effects play a central role in the innate immune defense. The complement system directs its activity specifically to the surface of invading microbes and injured cells. However, intact host cells which express endogenous complement regulators at the surfaces, or recruit fluid phase complement regulators to the surfaces are protected from complement attack. These regulators keep the whole complement activation under control and protect hosts against complement attack.

2.2.1.1 Complement activation

The alternative pathway

The alternative pathway represents a central immuno-surveillance system. The alternative complement activation is initiated in a rapid, spontaneous and antibody independent manner [11]. The key component of the alternative pathway is C3 which is a 185 kDa large molecule with a concentration of 1~2 mg/ml in serum. The first step of the alternative complement pathway activation is a spontaneous hydrolysis of the internal thioester bond of C3 by a "tick-over" process. Approximately less than 0.5% of C3 are spontaneously and continuously converted into a hydrolyzed form C3(H_2O), which has many characteristics of C3b [12]. C3(H_2O) binds Factor B in solution and forms a C3(H_2O)-Factor B complex, which is then converted into C3(H_2O)Bb and Ba by Factor D. The newly formed complex C3(H_2O)Bb represents the initial C3 convertase [13]. This fluid phase C3 convertase further cleaves C3 into the anaphylatoxin

C3a and C3b. The newly generated C3b covalently bind to the nearby surfaces via the thioester bond. This initial surface attached C3b recruits Factor B in the presence of Mg^{2+}, which is again cleaved by Factor D. The formed complex C3bBb is the alternative C3 convertase. Factor P in plasma binds to and stabilizes this C3bBb complex, thereby extending the lifetime of the active C3 convertase. The C3 convertase cleaves additional C3 into C3b and C3a. In this process, the cleavage products C3b expose an unstable, internal thioester bond based on the conformational change, which provides a nascent binding site within C3b for interaction with adjacent nucleophils (**Figure 4**). These surface attached C3b fulfill several functions: (i) formation of new C3 convertases and induction of a positive amplification loop of the alternative pathway complement cascade; (ii) binding to C3bBb to form a C3bBbC3b complex, which represents a C5 convertase, thereby initiating the terminal complement pathway and generating C5a; and (iii) opsonization onto the cell surfaces and promotion the further adhesion and phagocytosis by human phagocytes.

The lectin pathway

The lectin pathway is another antibody-independent route for complement activation, which is initiated by binding of the *mannose binding lectin* (MBL) or serum ficolins to oligosaccharides at microorganisms surfaces, apoptotic as well as necrotic cells [14-16] (**Figure 4**). MBLs and ficolins have structural similarity to C1. Both have globular binding regions that are associated with two serine proteases, named *mannose binding lectin associated protease* 1 (MASP1) and MASP2. These proteases are homologous with C1s and C1r molecules of the classical pathway [15,17]. MBL binding to a surface induces MASP1 to cleave MASP2. Such activated MASP2 cleaves C4 into C4b and C4a [18,19]. C4b attaches to a surface and couples C2, which is then cleaved by MASP2 into C2b and C2a. Finally a C3 convertase (C4bC2b) is formed, which will cleave the central component C3 into C3b and C3a. The newly generated C3b is further used for different purpose as described for the alternative pathway.

The classical pathway

The classical complement pathway activation is mainly initiated by binding of C1q to antigen-bound IgG or IgM and also to other activating structures, such as markers of damage cells, pentraxins and bacterial lipopolysaccharide [20-23]. C1 is a large hetero oligomeric complex with a molecular weight of approximately 800 kDa. This large complex is composed of single C1q molecule and two C1s and two C1r molecules. The intact C1q molecule consists of three subunits joined together. Each subunit contains a C-terminal globular head with

immunoglobulin receptor site, N-terminal collagen like sequences and the connected triple helix. These molecules of C1 are associated noncovalently in a Ca^{2+}-dependent manner [24]. Binding of the globular head C1q to different activating structures (for example, to IgG in immune complexes) induces a conformational change of the C1 complex. This triggers the activation of one enzymatic part of the C1 complex, C1r, which then cleaves the other two C1s molecules into the active serine esterase [25]. The active C1s cleaves the thioester-containing protein C4 into C4b and C4a. C4b is immediately attached to nucleophilic amine hydroxyle group at nearby surfaces. Actually, only about 10% of active C4b binds to the proteins or carbohydrates, the remaining molecules are bound to adjacent water, and form iC4b, which is then rapidly catabolized. Surface attached C4b recruits a zymogen C2. When combined with C4b, C2 becomes a substrate for C1s and is then cleaved into C2b and the small molecule C2a. The newly originated complex C4bC2b represents the classical pathway C3 convertase, which cleaves the central component C3 into C3b and C3a. The newly formed C3b is either integrated into the alternative complement pathway which induces an amplification loop of complement activity, or deposits onto the surfaces and mediates phagocytosis, or couples onto the C3 convertases to form C5 convertases and initiates the terminal complement pathway (**Figure 4**).

The terminal complement pathway

Following the activation, all three complement pathways merge at the central step, the cleavage of C3 into C3b and C3a. When newly generated C3b molecules are coupled with the C3 convertases (C3bBb or C4bC2b), C5 covertases are therefore generated. The newly generated C5 convertases further cleave C5 into C5a and C5b, which is the first step toward the *terminal complement pathway* (TP) [26]. The newly generated C5b is attached to C3b within the C5 convertases, binds C6 and forms a C5bC6 complex. Binding of C6 to C5b stabilizes the membrane binding site in C5b and exposes a binding site for C7. Binding of C7 to C5bC6 induces the transition of a hydrophilic to a hydrophobic state that allows the C5bC6C7 complex to bind tightly to the lipid bi-layers. Subsequently, C8 is coupled to the former complex, then followed by a polymeric complex of up to fourteen C9 monomers to form a *terminal complement complex* (TCC) [27,28]. TCC is therefore leads to the lysis of Gram negative bacteria or damaged host cells [29] (**Figure 4**). TCC is suggested to show additional different functions, such as inducing cytokine release, mediating cell adhesion and migration [30,31].

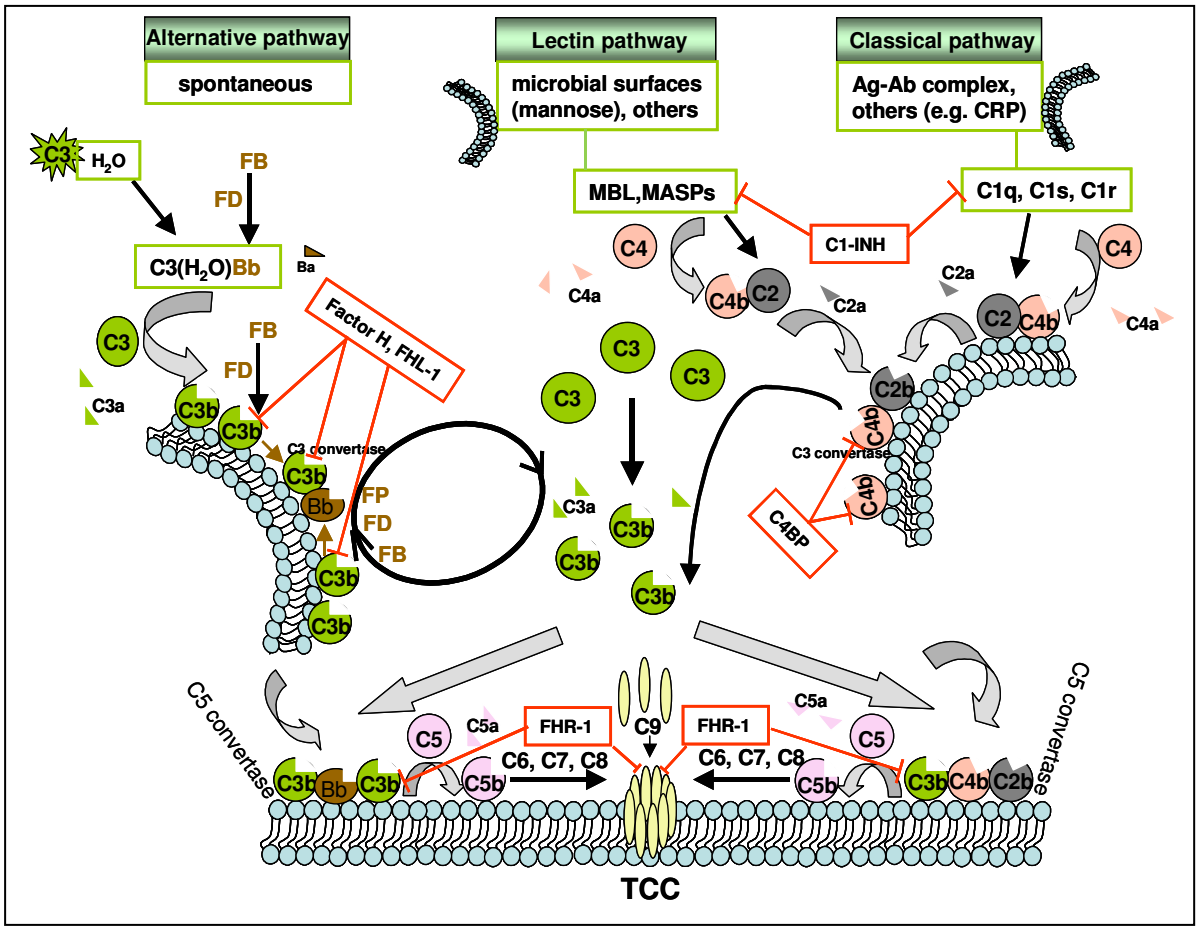

Figure 4. Complement activation pathways. Complement is activated by the alternative, lectin and classical pathways. Each pathway creates a C3 convertase which cleaves the central complement component C3 into C3a and C3b. C3b attached to nearby surfaces can be used for: (i) initiation of an amplification loop of the complement cascade via the alternative pathway; (ii) opsonization of surfaces for phagocytosis; and (iii) binding to the C3 convertases, which form C5 convertases. C5 convertases cleave C5 into C5a and C5b. C5b binds C6 and C7, followed with C8 and C9 to form the TCC. C3a and C5a initiate and amplify inflammation. Complement activation is highly regulated by complement regulators to protect host cells from complement attack. Alternative pathway activation is mainly regulated by the soluble complement inhibitors Factor H and FHL-1, whereas the classical and lectin pathway are mainly controlled by C1 inhibitor (C1-INH) and C4BP. C5 convertases and terminal complement pathway are controlled by CFHR1, vitronectin and clusterin. FB, Factor B. FD, Factor D. FP, Factor P.

2.2.1.2 Biological effects of the complement system

The complement system is an effective part of the innate immunity. This protein-based defense system serves multiple functions, such as: (i) initiation and amplification of an inflammation; (ii) induction or mediation of phagocytosis; (iii) elimination of the invaded microbes or clearance of the debris from tissues to maintain tissue homeostasis; and (iv) a link between the innate and adaptive immunity [9,10]. All these effector functions are based on newly generated cleavage products upon the activation of the complement system, such as C3a, C5a, C3b, iC3b, C3dg and TCC.

C3a and C5a, known as anaphylatoxins are generated and released into the fluid phase upon C3 and C5 cleavage by convertases. C3a and C5a bind to specific receptors, i.e. the C3a receptor, the C5a receptor and the C5a like receptor 2. Via ligation, C3a and C5a mediate leukocyte chemotaxis, adhesion of leukocytes to vascular endothelium, degranulations of the mast cells, contractions of the smooth muscles, increasement of vascular permeability, and an aggregation of platelets and leukocytes [32]. Recent findings suggest that C3a and C5a do also regulate cell apoptosis, lipid metabolism, recruit and activate antigen-presenting cells and T cells [33]. Furthermore, C3a displays an anti-microbial activity [34,35] (**Figure 5**).

C3b, C4b and the further degradation products iC3b or C3dg, which deposit onto target cells, are recognized by specific receptors, like *complement receptor type 1* (CR1, also named CD35), CR2 (CD21), CR3 (CD11b/CD18), CR4 (CD11c/CD18) that are expressed at different host cell surfaces (**Table 1**) [8]. The specific ligand-receptor interaction facilitates many biological effects. For example, binding of C3b or C4b to CR1 mediates endocytosis and phagocytosis of target cells by phagocytes and also mediates the transport of immune complexes to the liver for clearance. This complement dependent clearance of immune complexes prevents the chronic inflammation and complex deposition in blood vessels [36]. Moreover, CR1 binds C3b tagged immune complexes and subsequently inhibits B-cells to secrete antibodies, which is important for the down regulation of a B-cell response and blockade of auto antibody release [37]. In addition, binding of iC3b or C3dg to CR2 results in B cell activation and further induction of the adaptive immunity [38]. Binding of iC3b to CR3 efficiently mediates phagocytosis of opsonized microbes or particles by host phagocytes [39,40] (**Figure 5**). These complement dependent biological function lead to efficient elimination of any invading microbe and maintain host cellular homeostasis.

Table 1. complement receptors for C3 and C4 fragments

receptors	major ligands	cellular distribution
CR1 (CD35)	C3b, C4b	B cells, neutrophils, monocytes, macrophages, erythrocytes, follicular dendritic cells, glomerular epithelial cells
CR2 (CD21)	iC3b, C3dg	B cells, follicular dendritic cells, epithelial cells of cervix and nasopharynx
CR3 (CD11b/18)	iC3b	neutrophils, monocytes, macrophages, NK cells and follicular dendritic cells
CR4 (CD11c/18)	iC3b	neutrophils, monocytes, tissue macrophages

TCC, which is formed during the final phase of the complement activation cascade, mediates the lysis of the targeted cells and particles, like Gram negative bacteria and other foreign cells [29]. However, in recent years, it was reported that *soluble TCC* (sTCC) also induces cytokine release by endothelial cells. Based on the cytokine release, sTCC indirectly mediates adhesion and transendothelial migration of *polymorphonuclear leukocytes* (PMNs) [30]. In addition, TCC is suggested to induce phagocytosis of microbes by PMNs via depositing on the microbial surfaces [31] (**Figure 5**).

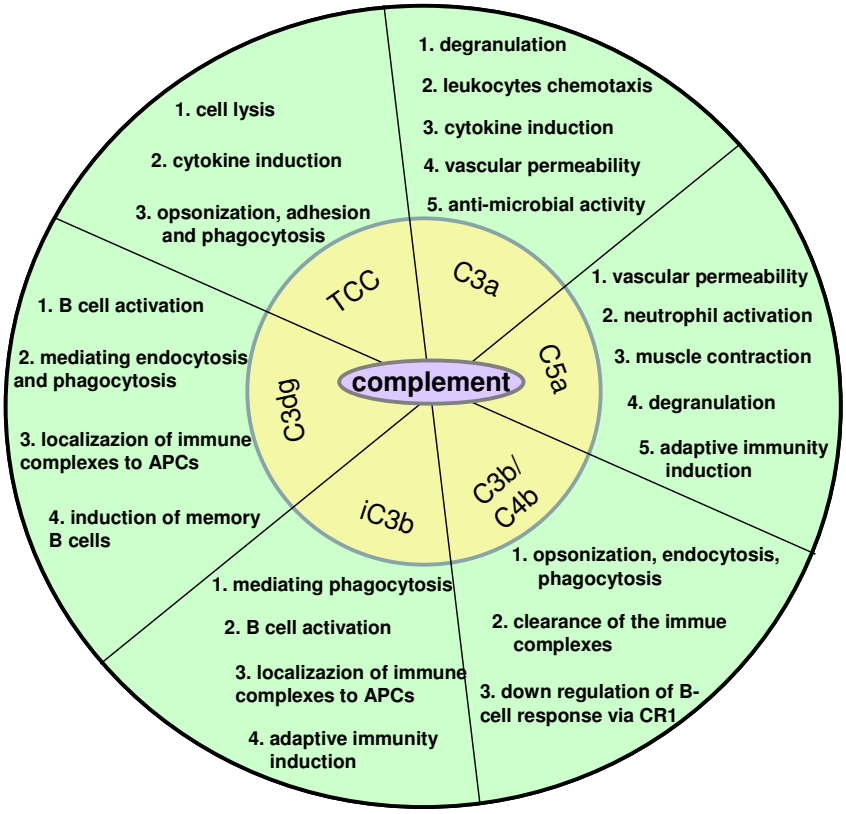

Figure 5. Complement based biological effects. The complement based biological effects are mediated by different cleavage products generated upon complement activation.

2.2.1.3 Complement regulation

Complement system is a very powerful protein-based defense system with potent pro-inflammatory effects. Uncontrolled complement activation can cause severe damage on autologous cells and tissues. Complement regulators protect host cells and extra-cellular matrices from complement attack and switch off the self amplifying cascade. These regulators are distinguished into plasma proteins and membrane bound proteins. Both types belong to the members of the complement activation regulator gene clusters. These regulators control complement activation at several steps of the whole cascade.

Fluid phase regulators

The Factor H protein family comprises eight homologous plasma proteins: Factor H, Factor H splicing variant *Factor H like protein 1* (FHL-1), and the *complement Factor H related proteins* (CFHR1-CFHR5) (**Figure 6**). The individual complement Factor H family protein shares common features, and each is composed of single structural elements termed *short consensus repeat domains* (SCRs). All members of the Factor H protein family are primarily produced in the liver. Factor H and FHL-1 are two major regulators of the alternative complement pathway that act both in fluid phase and on cell surfaces. Factor H is a 150 kDa plasma protein with a concentration of 350~600 µg/ml. Factor H is a single polypeptide, consisting of 20 SCRs (**Figure 6**). Each SCR has approximately 60 amino acids with four conserved cysteine residues which are responsible for initiating a globular domain folding [41,42]. FHL-1 is a splicing variant of Factor H, which is composed of the identical N-terminal SCRs1-7 of Factor H and four additional unique C-terminal amino acids. The plasma concentration of FHL-1 is approximately 50 µg/ml. Both regulators have three major functions: (i) cofactor activity for the plasma protease Factor I mediated C3b inactivation; (ii) inhibition on the alternative pathway C3 convertase assembly; and (iii) decay accelerating activity on the C3 convertase. All these regulatory functions of Factor H and FHL-1 are performed by the N-terminal SCRs1-4. Via binding to the regulatory region of Factor H, C3b is able to expose its cleavage site for Factor I. The α-chain of C3b is then cleaved by Factor I into the *inactive C3b* (iC3b) and C3f. This step controls or interrupts the complement cascade [43]. In addition, SCRs1-4 compete with Factor B for C3b binding, which inhibits C3 convertase assembly and accelerates the decay of an assembled C3 convertase. Factor H protects host cells against the alternative pathway complement attack by binding to cell surfaces. In addition to the regulatory regions, Factor H also contains multiple other ligand binding and functional regions, which are located either in the middle region or in the C-terminus. Single SCR7 domain binds heparin and CRP. SCRs8-11 represent the second CRP binding region [44] and SCRs12-15 are the second C3b binding sites. The C-terminal SCRs18-20 contain the binding regions for host surfaces, C3b and heparin (**Figure 6**) [43,45]. Recently, SCRs19-20 were identified as a new CRP binding region [46]. Mutations in the binding and functional regions of Factor H have severe effects on hosts and are associated with several diseases. For example, a mutation within the regulation domains (SCRs1-4) leads to the autoimmune disease, like *membranoproliferative glomerulonephritis II* (MPGN) because of the uncontrolled complement activity in the body [47]. A single amino acid polymorphism mutation in SCR7 is associated with *age related macular degeneration* (AMD) [48,49]. Mutations in the C-terminus induces autoimmune diseases like

atypical hemolytic-uremic syndrome (aHUS), which is due to the impaired complement control especially on endothelial cells and extracellular matrices [50-52].

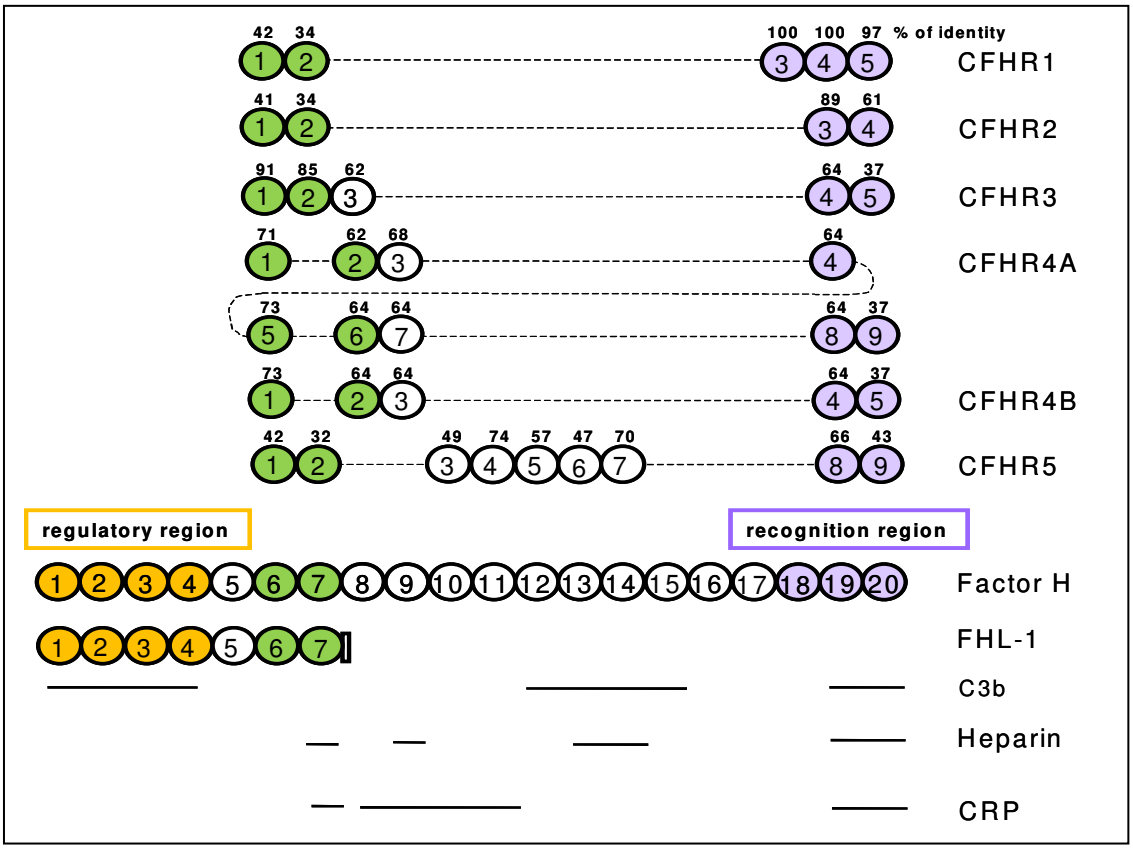

Figure 6. Domain structures of the Factor H protein family. Factor H protein family contains Factor H, FHL-1 and CFHR1-CFHR5. Factor H is composed of 20 SCRs. Each SCR contains approximately 60 amino acids. FHL-1 contains seven SCRs which are identical to the N-terminus of Factor H and four extra amino acids. CFHR1 contains five SCRs, which have different sequence homologous identities with Factor H. Factor H and FHL-1 are complement regulators of the alternative complement pathway. Both regulators have multiple binding and functional regions as labeled in the figure. CFHR1 is a C5 convertase and terminal pathway complement regulator.

C1 inhibitor and C4b-binding protein are two fluid phase complement regulators that control the classical and the lectin pathway. *C1 inhibitor* (C1-INH) is a serine protease inhibitor that binds activated C1 and removes C1r and C1s from the complex, thereby inhibiting the enzymatic activity to cleave C4. Similarly, C1-INH also blocks the activation of MASPs. Thus, C1-INH disables the first step of the classical and lectin pathway complement activation [53].

C4b-binding protein (C4BP) controls the later stages of the classical and lectin pathway. C4BP is a heptameric plasma regulator with a "spider-like" configuration. C4BP is a 570 kDa plasma glycoprotein, with a serum concentration of ca. 250 mg/l. The major form of C4BP consists of seven identical α-chains (70 kDa) and one single β-chain (45 kDa) [54]. Each α-chain

contains eight SCRs, while β-chain contains three SCRs (**Figure 7**). C4BP binds C4b via SCRs1-2 and binds heparin via SCRs1-3. By binding to C4b, C4BP makes C4b accessible for Factor I cleavage and inactivation. Furthermore, C4BP enhances the decay of the classical and lectin pathway C3 convertase [55]. C4BP also has low cofactor activity for cleavage of C3b and a low decay accelerating activity on the alternative pathway C3 convertase [56].

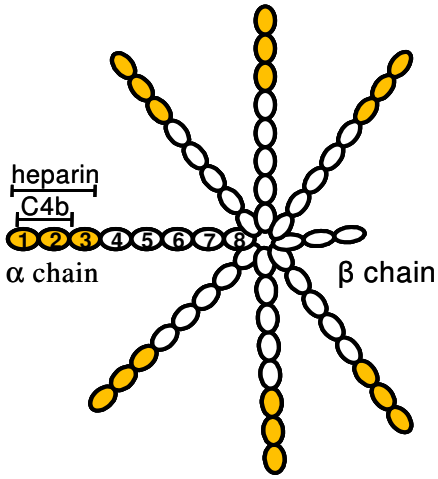

Figure 7. Domain structures of C4BP. C4BP contains seven identical α-chains (each has eight SCRs) and one single β-chain (three SCRs). C4b binding domains are located between SCR1 and SCR2, while heparin binding sites are SCRs1-3.

Complement Factor H related protein 1 (CFHR1) is a newly described soluble regulator of the terminal complement pathway [57]. CFHR1 is present in plasma as two different glycosylated forms (CFHR1α and CFHR1β). CFHR1 contains five SCRs (**Figure 6**). The C-terminal SCRs3-5 show high identity to SCRs18-20 of Factor H. Consequently, CFHR1 competes with Factor H for binding to cell surfaces. In contrast to Factor H, CFHR1 inhibits two steps of the terminal pathway cascade: (i) blocking the cleavage of C5 into C5b and anaphylatoxin C5a by alternative C5 convertase; and (ii) inhibition of binding of C7 to C5bC6, thereby blocking TCC formation.

Vitronectin and clusterin are two soluble regulators of the terminal complement pathway. Vitronectin exists in the plasma either as a single chain (75 kDa) or as a truncated form of 65 kDa and 10 kDa. Vitronectin inhibits the insertion of C5b-C7 complex into the cell membranes by binding to its membrane binding sites, thereby blocking the cell lysis [58]. In addition, vitronectin blocks the tubular formation by binding to TCC [59]. Clusterin is a 70~80 kDa glycoprotein, which binds the C5b-C7 and C5b-C8 complexes and prevents insertion of such complexes into cell membranes [60,61].

Membrane bound regulators

In contrast to the fluid phase inhibitors, there are also membrane bound regulators, *like decay accelerating factor* (DAF/CD55), *membrane cofactor protein* (MCP/CD46), and *membrane glycoprotein complement receptor 1* (CR1/CD35). DAF is expressed on vascular endothelial cells, erythrocytes, epithelial cells, lymphocytes and also on epithelial cells of the placental trophoblast. As a membrane regulator, DAF dissociates both C3 and C5 convertases of all three activation pathways. MCP is expressed on many kinds of cell types including epithelial and endothelial cells, fibroblasts, spermatozoa and placental trophoblasts and also circulating nucleated cells, but not erythrocytes. MCP is a cofactor for Factor I mediated inactivation of C3b and C4b. CR1 is expressed on circulating cells including erythrocytes. CR1 inhibits the assembly of the classical and alternative C3 convertases and also acts as a cofactor for Factor I mediated cleavage of C3b and C4b [62]. Furthermore, CD59, as a terminal complement pathway inhibitor, is a GPI anchored protein, which is mainly expressed on epithelial, endothelial and circulating cells [63]. CD59 regulates terminal complement pathway activity by blocking binding of C9 to C5b-C8 and C9 polymerization.

2.2.2 Cellular response of innate immunity

In addition to complement, cellular immune response is another major defense mechanism that protects hosts against infectious agents. Once microbial pathogens penetrate or cross the epithelial barriers, cellular immune response is initiated due to the recruitment of leukocytes like neutrophils, macrophages, dendritic cells and eosinophils to the sites of infection. There are three major stages in leukocytes migration: (i) attachment of circulating cells to the vascular endothelium by binding to the adhesion molecules; (ii) movement through the endothelial cells; and (iii) migration of leukocytes to the sites of infection or inflammation after traversing the endothelium [8]. This migration process is partially guided by a variety of soluble immune effector molecules. These effector molecules, also termed “messenger molecules” are cytokines or chemokines which are produced by local resident macrophages, endothelial cells and other cell types which is involved in response to microbial products. In addition, these effector molecules include anaphylotoxins C3a and C5a generated upon complement activation. Neutrophils are the most abundant populations of circulating white blood cells. Neutrophils are recruited and attracted at the earliest phase of inflammatory response [64]. Upon arrival at sites of infection, neutrophils immediately recognize invaded microbes via surface expressed PRRs. The PRRs-PAMPs ligation induces the effector function, such as phagocytosis. Following phagocytosis, the active neutrophils produce special

molecules, like *reactive oxygen species* (ROS), *nitric oxide* (NO) and proteolytic enzymes within a phagolysosome, thereby killing phagocytosed microbes. In addition, neutrophils contribute to collateral tissue damage that occurs during inflammation [65,66].

Following the early recruitment of neutrophils, monocytes migrate from blood into tissues where monocytes mature and differentiate to macrophages. Macrophages, similar to neutrophils, phagocytose and intracellularly kill microbes. Macrophages are also capable of extra-cellular killing of infected or altered self cells. Furthermore, macrophages produce growth factors for fibroblasts and endothelial cells that participate in the remodeling of tissues after infection and injury. Activated macrophages can also act as antigen-presenting cells, which are required for the induction of adaptive immunity [67].

In addition, these activated neutrophils and macrophages release additional cytokines and chemokines to recruit more macrophages, neutrophils and other leukocytes to the sites of infection. These newly activated leukocytes further phagocytose and kill more infectious agents. Thus, this whole defense system is efficiently amplified.

During the course of cellular immune response, many other human cells, such as dendritic cells, *natural killer* (NK) cells , eosinophils, basophils and mast cells also recognize and eliminate infectious microbes. For example, dendritic cells are often in contact with the external environment. Therefore, these cells are very important in engulfing and presenting antigens to cells of the adaptive immune system, therefore serving as a link between the innate and adaptive immune system [68]. NK cells are stimulated by IL-12 which is released via macrophages. The stimulated NK cells function as linage cells related to lymphocytes that recognize and direct kill infected or stressed host cells, secret inflammatory cytokines. In addition to killing infected cells, NK cells are the main source of *Interferon-γ* (IFN-γ) which activates macrophages to kill ingested microbes [69].

2.3 Adaptive immunity

The adaptive immune system is characteristic of exquisite specificity for distinct antigens and ability to remember repeated microbes. It is triggered in vertebrates when a pathogen evades the innate immune system and generates a threshold level of antigens. The major functions of the adaptive immunity include: (i) recognition of specific “non-self” antigens during the

process of antigen presentation; (ii) generation of responses that are tailored to maximally eliminate specific microbes or microbes-infected host cells; and (iii) development of immunological memory, in which each microbe is "remembered" by a signature receptor.

The adaptive immune response involves two groups of cells: lymphocytes and *antigen presenting cells* (APCs). Lymphocytes, a type of white blood cells, are produced in the bone marrow during the process of hematopoiesis and residue in various lymphoid organs. Lymphocytes are able to recognize newly generated antigens via specific membrane receptors. There are two major types of lymphocytes which differ in antigen recognition and functions: B lymphocytes and T lymphocytes.

2.3.1 B lymphocytes

B cells mature within the bone marrow, circulate in the blood and lymph system, then reside in different lymphoid organs. During the maturation, B cells express a unique antigen-binding membrane receptor, which are antibodies. When a B cell first encounters an antigen which is specific for the membrane bound antibody, the antigen attaches to a receptor. This complex therefore stimulates B cells to differentiate into effector cells called plasma cells and memory cells. Memory B cells display memory and "remember" that specific antigen. Helper T cells ($CD4^+$) help B cells in such differentiation and proliferation process [70]. Plasma cells survive for a few days and continuously secrete antibodies that are specific to the antigens. This process is very efficient as a single plasma cell can secrete approximately 10000 antibodies per second. Secreted antibodies are major effector molecules of humoral immunity, which combat microbes mainly by: (i) inducing classical complement activation via binding of IgG or IgM to microbes; (ii) mediation of phagocytosis by coating the microbes with IgG; and (iii) binding and neutralizing the microbes [71]. Memory B cells have a longer lifespan and continue to express membrane-bound receptor with the same specificity as the original parent cells. Whenever encountering the same antigen again, memory B cells rapidly recognize the antigen, multiply, change into plasma cells, and produce antibodies. This second exposure makes the response quick and more effective.

2.3.2 T lymphocytes

T cells arise from hematopoietic stem cells in bone marrow and migrate to the thymus to mature. During the maturation, T cells express a unique membrane receptor for antigen, called *T cell receptor* (TCR), which is distinguished from the one on other lymphocytes. T cell receptors are heterodimers, composed of two protein chains, either alpha and beta ($\alpha\beta$), or

gamma and delta (γδ). These two protein chains are linked by disulfide bonds. TCRs, in general, are responsible for recognizing antigens only in association with cell-membrane proteins known as *major histocompatibility complex* (MHC) molecules. T lymphocytes are mainly classified into T helper (T_H) cells and T cytotoxic (T_C) cells, which play a central role in cell-mediated immunity. When T cells encounter an antigen that is associated with a MHC molecule on a cell surface, T cells proliferate and differentiate into memory T cells and various effector T cells. However, effector T cells derived from T_H cell ($CD4^+$) and T_C cells ($CD8^+$) function differently. An effector T_H cell secrets various growth factors known as lymphokines, which play an important role in activating B cells, T_C cells, phagocytes and various other cells that participate in immune responses. Under the influence of lymphokines secreted by effector T_H cells, T_C cells are activated into effector cells called *cytotoxic T lymphocytes* (CTLs) via binding to a MHC class I coupled antigen [72]. CTL plays a vital function in monitoring host cells and eliminating any cells that display foreign antigen on MHC-I, such as microbe-infected host cells, tumor cell and cells from a foreign tissue.

Functionally, T helper lymphocytes can be further classified into T_H1, T_H2 and T_H17 which secrete different cytokines to facilitate a different type of immune response. For example, T_H1 lymphocytes produce proinflammatory cytokines (such as IFN-γ) which initiate and promote an inflammation. However, T_H2 lymphocytes produce anti-inflammatory cytokines (such as IL-10) which suppress and neutralize an inflammation. Different kinds of cytokines produced by Th1 and Th2 cross-regulate each other. Therefore, there is a balance of Th1 and Th2 responses [73,74].

In addition to T_H and T_C cells, a regulator T cell is the third type of T cells, formerly known as suppressor T cells. Regulator T cells are crucial for maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in thymus [75].

2.4 Epithelial and endothelial cell barriers

An epithelium is a tissue composed of cells that line the cavities and surfaces of structures throughout the body. Epithelium is often defined by the expression of the adhesion molecule, like E-cadherin. In addition to sensation, selective absorption and excretion, epithelial cells

serve as physical barrier and perform very complex and vital activities to protect the underlying tissue and subendothelial matrices from microbial infection, mechanical injury, harmful chemicals and excessive water loss [76]. As for interfering with the entry of microbes, epithelial cells provide a physical barrier, produce anti-microbial peptides and harbor intraepithelial lymphocytes that can kill microbes and infected cells. The epithelial barrier is considered as an innate immune defense.

An endothelium is a thin layer of cells that line the interior surface of blood vessels, forming an interface between circulating blood and the rest of the vessel wall. Endothelial cells are involved in many aspects of vascular biology, including: (i) barrier function; (ii) control of blood pressure; (iii) blood clotting; (iv) atherosclerosis; (v) formation of new blood vessels; and (vi) inflammation [77]. As for the barrier function, the endothelial cells provide a physical barrier that separates blood from tissues and prevents pathogenic microbes entering into the tissue from the blood stream, thereby protecting hosts from severe systemic infections. The endothelial cell barrier also controls the passage of materials and the transit of white blood cells into and out of the bloodstream [78].

2.5 Infection strategies of human pathogenic microbes

Upon spreading and disseminating into a host tissue or target cell, pathogens encounter different types of host cells, extra-cellular matrices, tissue fluid and blood which harbor the whole host defense system (physical tissue barriers, humoral and cellular immunity). In order to survive and overcome host immune surveillances and tissue barriers, pathogenic microbes have evolved sophisticated infection strategies, which include two major steps, immune evasion and tissue invasion.

2.5.1 Immune evasion

2.5.1.1 Escaping from complement attack

Complement forms the first defense line against infectious microbes and is considered as a central immuno-surveillance system of innate immunity. Complement evasion by pathogens represents a major threat for host immune protection. Strategies utilized by pathogens for complement evasion are mainly classified into: (i) acquisition of host complement regulators; (ii) expression of endogenous complement inhibitors; and (iii) inactivation of the complement effective components by proteolytic degradation.

Acquisition of host complement regulators is a common strategy used by many microbial pathogens for complement evasion. Many pathogens, including fungi, Gram negative and Gram positive bacteria, parasites and viruses recruit soluble human complement regulators (like Factor H, FHL-1 and C4BP) to their surface via specific surface virulence factors, which are called *Complement Regulator Acquiring Surface Proteins* (CRASPs) [79] (**Table 2**). Attached to pathogen surfaces, these human regulators maintain their inhibitory function and block complement activation at the level of the C3 convertases, thereby allowing the pathogen to control and evade host complement attack. In addition, some pathogens also utilize terminal pathway inhibitors, such as CFHR1, vitronectin and clusterin to block the TCC formation [80] (**Table 2**) . Furthermore, the recruitment of complement regulators is not only restricted to soluble, but also extended to membrane bound proteins. For example, *E. coli* and *Helicobacter pylori* utilize CD59 to interfere the TCC formation for terminal complement pathway evasion [81,82].

Expression of endogenous complement inhibitors represents a second mechanism used by pathogenic microbes to inactive host complement attack. For example, *S. aureus* expresses *extra-cellular fibrinogen binding protein* (Efb) and *its homologous protein* (Ehp) that bind C3 and further inhibit the substrate C3 conversion by the C3 convertases, and also expresses SCIN that binds and stabilizes the assembled C3 convertases, thereby interrupting further complement progression [83-88]. In addition, *S. aureus* expresses the *chemotaxis inhibitory protein* (CHIPS) that binds and neutralizes the C5a receptor on the surfaces of neutrophils and monocytes, thus blocking the anaphylotoxin C5a mediated chemotaxis [89-91]. *B. burgdorferi* expresses a CD59-like protein on the surface, which binds C8 and C9, and thus inhibits TCC formation. *Streptococcus pyogenes* strains M1 and M57 express *streptococcal inhibitor of complement* (SIC) which inhibits TCC formation by preventing the uptake of the C5b-C7 onto the cell membranes [92]. In addition, *herpes simplex virus* type 1 and 2 use the transmembrane protein gC1 and gC2 to bind C3b, thus accelerating the decay of the alternative pathway C3 convertase [93]. gC1 also inhibits the interaction of C3b with C5 and Factor P [94]. *Schistosoma* and *Trypanosoma* parasites express complement C2 receptor trispanning protein, which disrupts the interaction between C2 and C4, thus interfering with the classical pathway C3 convertase formation [95].

Expression of proteases to allow proteolytic degradation is an additional strategy utilized by microbial pathogens to inactive host complement attack. For example, the *streptococcal C5a peptidase* (ScpB) expressed by Group B *streptococci* cleaves and inactivates chemokine C5a, thereby providing a significant advantage for mucosal colonization. *C. albicans* produces secreted aspartic proteinases (Saps), like Saps 1, 2 and 3 which degrade C3b, C4b and C5, thereby blocking complement activation and effector function, such as C3b and C4b surface depositions [96]. In addition, *Pseudomonas spp.* proteases (PaE and PaAP) [97] and *Porphyromonas spp.* proteases (PrtH) [98] cleave C3 into non-functional fragments, thus inhibiting C3b deposition and complement activation at the bacterial surface.

2.5.1.2 Evasion from cellular immune responses

In addition to complement attack, infectious microbes encounter cellular immune responses mediated by different cell types. Therefore, microbial pathogens have evovled strategies to escape from these immune surveillances for an infection. According to mechanisms, evasion from the cellular immune responses can be further classified into four sub-types, (i) escaping from phagocyte responses; (ii) interfering with the cytokine release and blocking the cytokine-receptor interaction; (iii) interfering with the antigen presentation; and (iv) interfering with effector function of B and T cells. Examples and the exact mechanism for each type of the cellular immune evasion are listed below (**Table 3**).

2.5.2 Tissue invasion

In order to spread and cause invasive disease microbial pathogens aslo found ways to overcome tissue barriers including extra-cellular matrix, nonphagocytic host cells, like epithelial cells and endothelial cells (**Table 3**).

Table 2 Human complement regulators bound by pathogens for complement evasion

Pathogens	Microbial proteins	bound regulators			References
		AP	CP/LP	TP	
Fungi					
Candida albicans	*Gpm1*	Factor H, FHL-1			99-102
	?		C4BP		
	αvβ3 integrin-like protein			vitronectin	
Aspergillus spp.	?	Factor H, FHL-1	C4BP	CFHR1	103,104
Bacteria					
Actinobacillus actinomycetemcomitans	Omp 100	Factor H			105
Borrelial afzelii	BaCRSAP-1,2,3	Factor H, FHL-1			106-108
	BaCRASP-4,5	Factor H			
	?		C4BP		
Borrelia burgdorferi	BbCRSAP-1,2	Factor H, FHL-1			107,109-111
	BbCRSAP-3,4,5, HcpA	Factor H		CFHR1	
Borrelial Hermsii	FhbA	Factor H, FHL-1			112,113
	BhCRASP-1	Factor H		CFHR1	
Escherichia coli	Stx2	Factor H			81,114
	?			CD59	
Escherichia coli K1	?	Factor H			115,116
	OmpA		C4BP		
Haemophilus influenzae	?	Factor H, FHL-1			117-119
	?		C4BP		
	Hsf			vitronectin	
Leptospira interogans	LenA (LfhA)	Factor H		CFHR1	120,121
	LenB	Factor H			
Moraxella catarrhalis	UspA1,2		C4BP		122,123
	UspA2			vitronectin	
Neisseria gonorrhoeae	Por1A	Factor H			124,125
	Por1A, Por1B		C4BP		
Neisseria meningitidis	fHbp (GNA 1870)	Factor H			126-130
	Por A		C4BP		
Pseudomonas aeruginosa	Tuf	Factor H		CFHR1	131
Staphylococcus aureaus	Sbi	Factor H		CFHR1	132-134
	?			vitronectin	
	?			clusterin	
Streptococcus pyogenes	M protein	Factor H, FHL-1	C4BP		135-141
	Fba	Factor H, FHL-1			
	Scl1.6	Factor H		CFHR1	
	SIC			clusterin	
Streptococcus pneumoniae	Bac, Hic, PspC	Factor H			
	PspC 4.4		C4BP		142-146
	?			vitronectin	
Parasites					
Echinococcus granulosus	?	Factor H			147
Onchocerca volvulus	microfilari	Factor H			148
Virus					
HIV	gp41, gp120	Factor H			149
Westnilvirus	NS1	Factor H			150

Table 3 Immune evasion and tissue invasion of microbial pathogens

Types	Mechanisms	Examples	References
Evasion of cellular immune responses			
Escape from phagocytes response	**1.Escaping PRRs recognition of phagocytes by shielding PAMPs**	***C. albicans*** **(β-glucan)** ***Histoplasma*** **(β-glucan)**	151-153
	2. Induction of programmed host cell death	***Salmonella*** ***Shigella***	154,155
	3. Persistent in the intracellular host phagocytes	***Listeria Monocytogenes*****,** ***Samonella*****,** ***Candida,*** ***Aspergillus***	156,157
	4. Shedding a decoy in a surrounding of pathogens	***Pneumocystis*** **(gpA)**	158
Modulation of the cytokine release	**1. Induction of anti-inflammatory cytokine release by favoring TLR2 activation, instead of TLR4**	***C. albicans*** ***A. fumigatus***	159,160
	2. Inducing anti-inflammatory cytokine IL-10 release, thus suppressing INF- γ production by antigen specific T cells and down-regulating host immune responses	***Cryptococcus neoformans*** **(Capsule);** ***Bordetella pertussis*** **(FHA)** ***Yersinia enterocolitical*** **(LcrV)**	161-164
Interfering with the antigen presentation	**1. Inhibition on C3d and CR2 interaction, thus preventing CR2-mediated B cell activation and maturation**	***S. aureus*** **(Efb and Ehp)**	165
	2. Diminishing the ability of antigen processing cells to degrade internalized antigens	***Helicobacter pylori*** **(VacA)**	166
	3. Inhibition on the MHC molecule surface expression on infected host cells	***Chlamydia trachomatis***	167
Interfering with effector function of T and B cells	**1. Inhibiting tyrosine phosphorylation of T and B cell receptors, thereby suppressing antigen-specific T cell activation and IL-2 release, and up-regulating of the costimulatory molecule CD86 after B cell receptor engagement with antigens**	***Yersinia pseudotuberculosis*** **(YopH),**	168,169
	2. Shedding proteins to block CEACAM1 on T cell surfaces, thereby inhibiting the activation and proliferation of $CD4^+$ T cells	***Neisseria gonorrhoeae*** **(OPA protein)**	170
Tissue invasion			
Extra-cellular matrix degradation	**1. Secretion or expression of lytic enzymes**	***C. albicans*****,** ***Pseudallescheria boydii***	171-173
	2. Recruitment of host plasminogen onto pathogen cell surfaces	***C. albicans*****,** ***Pseudomonas aeruginosa, Cr. neoformans*****,** ***Borrelia spielmanii,*** **group A** ***streptococci***	101,131,174-177
Invading non-phagocytic epithelial and endothelial cell barriers	**1. Induction of passive uptake by host cells**	***C. albicans*** ***A. fumigatus***	178-182
	2. Active penetration mediated by production of lytic enzymes	***C. albicans***	183-185
	3. Induction of membrane ruffling and projection of microvilli that surround the organisms and pull them into the cell	***Cr. neoformans***	186,187

2.6 *Candida albicans*

2.6.1 General properties

Candida albicans is one of the most important members of the yeast genus and is a causal agent of opportunistic superficial and systemic infections in humans. *C. albicans* has two different forms, yeast and hyphal forms. *C. albicans* has a thick cell wall, an outer structure which protects the fungus from environmental stress like osmotic pressure and defines the shape and physical strength of the fungal cell. The cell wall of *C. albicans* is a highly complex structure composed of glucans (β-1,3 and β-1,6 glucans), chitin, GPI-anchored protein and mannoproteins inserted in a network of polysaccharides (**Figure 8**). This fungal cell wall plays several important roles for *C. albicans* survival and initiating an infection: (i) first coming into contact with host cells; (ii) carrying important antigenic determinants; (iii) being responsible for the adherence of the pathogen to host surfaces; and (iv) establishing a cross-talk with hosts.

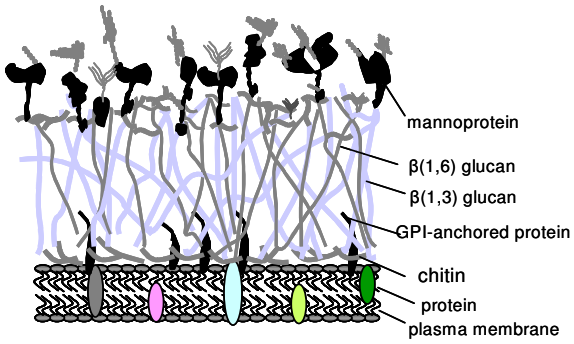

Figure 8. The cell wall of *C. albicans*

Under normal circumstances, *C. albicans* is a commensal fungus which usually resides as part of mucosal microflora (oral cavity, gastrointestinal tract or the vagina) [188]. About 80% of the human population carries *C. albicans* with no harmful effects. However, *C. albicans* can turn from a commensal yeast into an infectious pathogen especially in patients with some predispositions. These predispositions include: (i) the disrupted balance of the normal flora; (ii) impaired barriers; and (iii) immunosuppression which is due to cancer treatments, immunosuppressive therapy, long-term catheterization, use of broad spectrum antibiotics, steroids, or diseases such as AIDS. *C. albicans* infection ranges from superficial to systemic infection. Despite currently applied anti-fungal therapies, both mortality and morbidity mediated by infection are still unacceptably high [189-191]. More than 75% of people with systemic candidal infection die. Moreover, resistant *C. albicans* strains are continuously increasing in recent years. *C. albicans* is the most frequently isolated fungal pathogen from infected individuals. Blood, brain, eye, kidney, and heart are the organs that are most

frequently affected. In addition, lung, liver, and spleen can also be infected by *C. albicans*. Signs and symptoms of a candidal infection in patients vary depending on the location of the infection. Oral candidiasis, called thrush, is characteristic of thick, white lacy patches on top of a red base. Oral candidiasis may also make the tongue look red without the white coating. Vaginal yeast infection is a white cheesy discharge that typically itches and irritates the vagina and the surrounding outer tissues.

2.6.2 Virulence factors and evasion strategies of *C. albicans*

Upon the course of infection, *C. albicans* encounters host surfaces, the outer layer of the skin, tissue barriers as well as humoral and cellular immune responses. Therefore, virulence factors and evasion strategies utilized by *C. albicans* to initiate an infection consist of: (i) the ability to adhere and colonize onto host epithelial surfaces; (ii) tissue invasion by production or acquisition of tissue damaging enzymes, as well as morphogenesis or expression of surface invasion; and (iii) evasion of various immune surveillances, such as complement evasion and evasion of cellular response of the innate immunity.

2.6.2.1 Adhesion and invasion of the epithelium by *C. albicans*

Adhesion and colonization of *C. albicans* onto host epithelial cell is the first step for invasion and crossing the epithelium. This step is mediated by surface expressed adhesins. The adhesins are mostly mannoproteins. Both the protein and carbohydrate portions are involved in the adherence. For example, *Agglutinin-like sequence* (Als) family, *hyphal cell wall protein 1* (Hwp1) and *integrin-like protein 1* (Int1) mediate *C. albicans* in adhesion and colonization [192-194]. Hwp1, a developmentally regulated adhesin of germ tubes and hyphae, attaches to buccal epithelial cells by an unconventional, transglutaminase-mediated mechanism of adhesion [195,196]. In addition, transcription of several genes, such as *PHR1, PRA1, FKH1, TEF1 HYR1* and *EED1* is up-regulated during the oral infection [197,198]. During adhesion onto the host epithelial cell surfaces, *C. albicans* secretes proteases like Saps 9 that degrades and deactivates host anti-microbial peptide secreted by epithelial cells, thereby allowing *C. albicans* to avoid the anti-microbial attack on the epithelial cell surfaces [199]. Following adhesion, *C. albicans* induces the hyphal production and secretes multiple extra-cellular hydrolytic enzymes (e.g., Saps, Plb, and Lip families) that digest or destroy cell membranes, and degrade host surface molecules to further break down and enter host epithelial cell barriers [200]. In addition, *C. albicans* induces host epithelial cells to produce pseudopods that mediate the accumulation of epithelial cells to surround the microbes and pull *C. albicans* into the host cells [178]. Thus,

C. albicans can invade epithelial cells by both passive uptake and active penetration. However, these two routes are exploited to a different extent which depends on the types of host cells faced by the fungus [201]. During superficial infection, *C. albicans* is often hidden within host epithelial cells, which is suggested that the intracellular location protects *C. albicans* from the host immune attack, such as complement attack and phagocytosis [156].

2.6.2.2 Complement evasion of *C. albicans*

Following invading epithelial barriers, *C. albicans* gains access to the blood stream. However, *C. albicans* immediately encounters the complement response. To combat the complement attack, *C. albicans* captures host complement regulators Factor H, FHL-1 and C4BP from human plasma to its surface [79,99,100,103]. Attached to the surface, all these complement regulators retain complement regulatory functions, which allows *C. albicans* to evade immune and complement attack. The *phosphoglycerate mutase* (Gpm1p) is the first Factor H binding protein identified from *C. albicans* yeast cells. Gpm1p, also termed *complement regulator acquiring surface protein 1* (CRASP1) [101] mediates *C. albicans* acquiring Factor H and FHL-1 from NHS for immune and complement evasion. Gpm1p binds Factor H via two contact sites, SCRs6-7 and SCRs19-20, and binds FHL-1 via SCRs6-7. A Gpm1p deficient yeast mutant shows reduced Factor H binding, but not completely, which indicates that an additional Factor H binding protein exists at *C. albicans* surface. In addition, *C. albicans* binds C4BP, a classical pathway complement regulator. Binding domains within C4BP for *C. albicans* were mainly localized in SCR1 and SCR2 of the α-chain of C4BP [100]. However, which protein from *C. albicans* that binds C4BP still remains unknown.

Vitronectin is a terminal complement pathway inhibitor. The yeast form of *C. albicans* expresses a receptor, which is antigenically related to the host vertebrate αvβ3 integrin receptor, so called αvβ3 integrin-like protein. This integrin-like receptor acquires vitronectin to yeast cell surface to inhibit TCC formaion, thereby controlling the terminal complement pathway attack [202]. Some clinical isolates of *C. albicans* bind vitronectin [203], which shows an example that recruitment of vitronectin plays a role for *C. albicans* infection in an *in-vivo* situation. This binding is strongly inhibited by heparin, which indicates the involvement of the glycosaminoglycan-binding region of vitronectin in *Candida*-vitronectin interaction [204].

In addition to the immediate acquisition of host complement regulators for complement evasion, *C. albicans* produces Saps (such as Sap1, Sap2 and Sap3) that degrade and inactivate

human complement components C3b, C4b and C5, thereby interfering with the complement activation cascade and the effector functions [96].

2.6.2.3 Evasion of host cellular immune responses by *C. albicans*

In addition to complement attack, locally resident and newly attracted phagocytes are the next "enemy" for *C. albicans*. One strategy used by *C. albicans* is to shield the surface PAMPs to escape PRR recognition of the phagocytes. On *C. albicans* cell wall, β-glucan is a common conserved component which binds dectin-1 and favors recognition of *C. albicans* by human phagocytes. However, during the hyphal growth, *C. albicans* surface mannans shield β-glucan from recognition by dectin-1, thereby avoiding the phagocytosis by phagocytes [151,152].

In addition, cytokines are a group of immune effector molecules that are used for cellular communication in both the innate and the adaptive immunity. *C. albicans* uses multiple mechanisms to interfere with or modulate the cytokine release and cytokine-receptor interaction, thereby modulating cytokine-mediated host immune responses. For example, *C. albicans* hyphae is able to evade TLR4, but favor TLR2 activation. By avoiding TLR4 recognition, *C. albicans* inhibits Th1-response mediated pro-inflammatory cytokine release (like IFN-γ), thereby down-regulating the further host immune response mediated by pro-inflammatory cytokines. In parallel, *C. albicans*, by favoring TLR2 activation, initiates the TLR2 mediated Th2 response that induces an anti-inflammatory cytokine response, like IL-10, thereby inhibiting macrophage activation and oxidative burst [160,205,206].

If evasion from the recognition by host phagocytes fails, *C. albicans* inside phagocytes initiates NO-scavenging flavohemoglobin gene expression, induces an anti-NO defense mechanism, therefore converting NO to less toxic molecules [207]. Furthermore, *C. albicans* also actively suppresses the production and release of *reactive oxygen species* (ROS), thereby inhibiting killing or destruction of fungus by phagocytes [208]. In addition, *C. albicans* resists the intracellular killing and escapes from the phagocytes by slow growth, development of hyphal growth and alternative carbon utilization [209].

2.6.2.4 Adhesion and invasion to the endothelium by *C. albicans*

For a systemic and disseminated infection, *C. albicans* has to enter into the blood stream, spread through out the body, overcome the blood vascular endothelial cell and invade target organs. Generally, *C. albicans* utilizes three mechanisms for invasion of the epithelial cell

barriers: (i) phagocytosis of *C. albicans* by host leukocytes, which then dispense across the endothelial cell lining of blood vessels; (ii) the passage of *C. albicans* between the endothelial cells, which likely happens in vascular beds of organs like kidney; and (iii) the endocytosis of *C. albicans* by the endothelial cells via ligand-receptor interaction [210]. *Agglutinin-like sequence 3* (Als3) is an invasin expressed by *C. albicans* which mediates the endocytosis of the fungus into endothelial cells. Als3 binds to the N-cadherin expressed on endothelial cell surfaces. This interaction induces the phosphorylation of the endothelial surface proteins, thus causing the rearrangement of the endothelial cell microfilaments to produce pseudupods and initiate the edocytosis [182]. In addition, Hgc1 is a newly discovered *C. albicans* protein which mediates dynamic *C. albicans*-endothelium adhesion during circulation [211]. It also suggests that *C. albicans* by internalization into the endothelial cell hides from the detection by professional phagocytes [156].

2.6.2.5 Adhesion and invasion of extra-cellular matrix by *C. albicans*

Upon an infection, *C. albicans* also encounters extra-cellular matrices which mediate the cell assembled and bound together in tissues. In order to cross these impact barriers, *C. albicans* either secretes lytic enzymes (like Saps or lipases) [200], or acquires human plasminogen onto surface. Surface attached plasminogen is activated into plasmin by activators (uPA or tPA) and degrade extra-cellular matrices, thereby mediating *C. albicans* tissue invasion. Eight plasminogen binding proteins are expressed on *C. albicans* surface, such as alcohol dehydrogenase, thioredoxin peroxidase, catalase, transcription elongation factor, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase (Gpm1p) and fructose bisphosphate aldolase [101,212].

Besides these efficient evasion mechanisms, the biofilm formation is another virulence factor, which induces *C. albicans* resistant to a range of anti-fungal agents in current clinical use, including amphotericin B and fluconazole. Therefore, biofilm formation appears to be one of the multiple resistance mechanisms of *C. albicans* [213].

2.7 *Aspergillus fumigatus*

2.7.1 General properties

Aspergillus fumigatus is a fungus which belongs to the *Aspergillus* genus and is one of the most common *Aspergillus* species that cause diseases in immuno-compromised individuals. *A. fumigatus* is widespread in nature, typically found in soil and decaying organic matter, such as compost heaps. In nature, *A. fumigatus* plays an essential role in carbon and nitrogen recycling. Colonies of the fungus produce thousands of minute gray-green conidia that readily become airborne. Everybody inhales hundred of spores per day. These spores are quickly eliminated by host immune system in healthy individuals. However, in immuno-compromised individuals, such as organ transplant recipients and people with AIDS or leukaemia, the fungus becomes pathogenic, evade the host weakened defenses and cause a range of diseases, generally termed aspergillosis.

2.7.2 Virulence factors and evasion strategies of *A. fumigatus*

Similar to *C. albicans*, virulence factors and evasion strategies utilized by *A. fumigatus* to initiate an infection include: (i) the ability to adhere and colonize onto host epithelial surfaces; (ii) tissue invasion by production and acquisition of tissue damaging enzymes, as well as expression of surface invasion; and (iii) evasion of various immune surveillances, such as complement evasion and evasion of cellular response of the innate immunity.

2.7.2.1 Adhesion and invasion of the epithelium by *A. fumigatus*

Invasive aspergillosis is initiated by inhalation of conidia. These conidia are then deposited in the alveoli. Subsequently, the conidia adhere and invade the epithelial cell lining of the alveoli, which is a crucial step for establishment of aspergillosis. In this process, *A. fumigatus* induces its own endocytosis mainly by type II pseumocytes (great alveolar cells). Type II pseumocytes contact with *A. fumigatus* and produce pseudopods that engulf the fungus [180]. Once internalized by type II pseumocytes, the conidia translocate to the late endosome/lysosome. The internalized conidia remain viable for rather long periods compared to conidia within macrophages, and also limit the release of pro-inflammatory cytokines IL-6 and IL-8 [156,181]. Eventually conidia germinate and escape from the endosome, penetrate the plasma membrane and disseminate into deeper tissues.

2.7.2.2 Complement evasion of *A. fumigatus*

After overcoming the epithelial cell barrier, *A. fumigatus* encounters the complement attack. *A. funigatus* conidia acquire human complement regulators like Factor H, FHL-1 and CFHR1 for complement evasion. Conidia bind Factor H via N-terminal SCRs1-7 and SCR20, and bind FHL-1 via SCRs1-7. Surface bound Factor H maintains regulatory activity and assists Factor I to cleave C3b or dissociates the C3 convertase. This inhibitory effect therefore inhibits the complement activation and the immune effector function, like inflammation and phagocytosis. In addition, *A. fumigatus* hyphae produce a water-soluble factor that decreases C3b deposition on the fungal surface and interferes with the C3b mediated phagocytosis and killing of the fungus by human phagocytes [214]. Furthermore, *A. fumigatus* secrets proteolytic enzymes that degrade the central component C3, thereby blocking the complement attack [215].

2.7.2.3 Evasion of host cellular immune responses by *A. fumigatus*

Facing the cellular response, *A. fumigatus* induces hyphae formation to evade the TLR4 recognition, thereby inhibiting TLR4 mediated pro-inflammatory cytokine release, like IFN-γ and subsequent host immune response [216]. Similar to *C. albicans,* hiding inside the non-phagocytic cells is another strategy to escape from the phagocytic recognition. In addition, several fungal metabolites interfere with the phagocytosis. For example, aflatoxins depress phagocytosis, intracellular killing and spontaneous superoxide production by macrophages [217]. Gliotoxin inhibits macrophage adhesion and phagocytosis of the fungus in vitro without leading to cell death and prevents spleen cells from inducing alloreactive cytotoxic T cells [218]. In addition, *A. fumigatus* conidia release a “conidia inhibitory factor”, which binds the contractile elements of the cell cytoplasma, thereby blocking chemoattractant-induced PMNs migration [219-221]. *A. fumigatus* conidia also release *A. fumigatus diffusible product* (AfD), which inhibits the production of the pro-inflammatory cytokine transcription in rat alveolar marophages [222]. When phagocytosed by professional phagocytes, *A. fumigatus* is still able to resist the oxidants, or fails to stimulate the respiratory burst in macrophages [223,224].

2.7.2.4 Adhesion and invasion of the endothelium by *A. fumigatus*

Invasion of the blood vessels is a key feature of invasive aspergillosis [225,226]. *A. fumigatus* invade the vasculature and enter into the blood stream by passing from the abluminal to the luminal surface of the pulmonary endothelial cells, which leads to the disruption of the endothelial cell monolayers. After entering into the blood stream *A. fumigatus* hyphae

disseminates throughout the body. Finally, the hyphae penetrate the luminal surface of vascular endothelial cells via induction of its own endocytosis to invade host organs [180,227,228].

2.7.2.5 Adhesion and invasion of extra-cellular matrix

Intact *A. fumigatus* conidia adhere to extra-cellular matrix compounds like laminin, fibronectin, collagen type 1 and 4 and also to fibrinogen via surface expressed adhesin. The laminin binding protein of the fungus is found as a 72 kDa cell wall glycoprotein, called *extra-cellular thaumatin domain potein* (AfCalAp). Following adhesion, *A. fumigatus* produce lytic enzymes to degrade the extra-cellular matrices for tissue invasion. For example, an *extra-cellular elastinolytic alkaline proteinase* (AFAlp) is able to degrade collagen, fibrin, fibrinogen and elastin [229]. In addition, *A. fumigatus* also binds plasminogen and utilizes it to degrade the extra-cellular matrix [103] .

2.8 *Streptococcus pneumoniae*

Streptococcus pneumoniae is Gram-positive and belongs to the *Streptococcus* genus. As a significant human pathogenic bacterium, *S. pneumoniae* was recognized as a major cause of pneumonia in the late 19th century. Furthermore, this bacterial pathogen cause many other types of pneumococcal infection, including acute sinusitis, otitis media, meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess. *S. pneumoniae* is the most common cause of bacterial meningitis in adults and children, and is one of the top two isolates found in the ear infection, called otitis media. *S. pneumoniae* is part of the normal upper respiratory tract flora. However, *S. pneumoniae* is also opportunistic and can become pathogenic under the suppressed immune system. Pneumolysin (an anti-phagocytic capsule), various adhesins and immunogenic cell wall components are major virulence factors.

S. pneumoniae evolved many different evasion strategies for establishing an infection. For example, *S. pneumoniae* utilizes *Pneumococcal surface protein C* (PspC) to acquire Factor H onto the bacterial surface, which mediates complement evasion [230]. However, the effect of PspC on complement attack varies from the strain background and capsular serotype [231]. In addition, surface bound Factor H mediates *S. pneumoniae* adhesion to human epithelial cells [232].

2.9 Aim of the project

C. albicans is the most frequently isolated fungal pathogen from infected individuals. Infections with *C. albicans* range from superficial to systemic disorders [233]. Despite currently applied anti-fungal therapies, both mortality and morbidity mediated by *C. albicans* are still unacceptably high [189-191]. The aim of my PhD work was to identify and characterize central immune evasion proteins of this human pathogenic yeast *C. albicans*.

C. albicans utilize human complement regulators, Factor H, FHL-1 and C4BP for complement evasion. In order to identify and characterize *C. albicans* encoded proteins that bind human complement regulators, via screening an expression cDNA library, I identified the *pH-regulated antigen 1* (Pra1) as a fungal surface protein that binds Factor H and FHL-1. Pra1 which was originally identified as a fibrinogen binding protein, is a yeast surface protein and is also released [234,235]. The released Pra1 protein binds to the integrin receptor CR3 (CD11b/CD18; $\alpha_M\beta_2$) expressed on the surface of human leukocytes [235]. The transcription of *PRA1* gene is up-regulated upon co-culture of *C. albicans* with human epithelial and endothelial cells [236]. However, how Pra1 mediates *C. albicans* complement evasion; whether and how Pra1 mediates *C. albicans* contact with human cells; and whether and how Pra1 functions differently at different sites are still unknown. Therefore, the goals of this project was to define the different functions of Pra1 at the different locations, i.e. (i) at the yeast surface; (ii) as a secreted protein in fluid phase; and (iii) bound to the surfaces of human cells.

3. Overview of the inserted manuscripts

1. Immune evasion of the human pathogenic yeast *Candida albicans*: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein.
   Shanshan Luo, Sophia Poltermann, Anja Kunert, Steffen Rupp, Peter F. Zipfel
   Mol Immunol. 2009 Dec; 47(2-3):541-50.

   Major aspects of the manuscript

   In this manuscript, the pH-regulated antigen 1 (Pra1) is identified as a novel Factor H binding protein by screening a *C. albicans* cDNA expression library. Pra1 was recombinantly expressed and purified in *Pichia pastoris* system. *Candida* Pra1 is expressed on the surface and also released to culture supernatant. As a surface protein, Pra1 acquires two human complement regulators Factor H, FHL-1 as well as plasminogen, mediates complement evasion, and degradation of extra-cellular matrices. A Pra1 overexpressing strain with about twofold Pra1 level on the surface binds more Factor H and plasminogen. As a released protein, Pra1 enhances complement control of Factor H in direct vicinity of the yeast.

   Own contribution and contribution of the coauthors to the manuscript

   - Shanshan Luo has planed, performed, and interpreted the following experiments: expression and purification of Pra1 by FPLC, characterization of Pra1 anti-serum; ELISA and ligand affinity blotting analysis of Pra1 binding to Factor H, FHL-1 and plasminogen; localization of Pra1 binding domains within Factor H and FHL-1; functional assay of bound Factor H, FHL-1 and plasminogen; flow cytometry, confocal microscopy, Western blotting and *Candid*a ELISA for analyzing Pra1 location; comparison of Factor H and plasminogen binding to a Pra1 overexpressing strain with wild type strain; Shanshan Luo also wrote the manuscript.
   - Sophia Poltermann assisted in *Candida* ELISA and confocal microscopy assay.
   - Sophia Poltermann and Anja Kunert screened the cDNA library and identified Pra1 as a Factor H binding protein.
   - Steffen Rupp provided a Pra1 overexpressing strain.
   - Peter F. Zipfel designed the study, interpreted the results and wrote the manuscript.

2. Secreted pH-regulated antigen 1 of *Candida albicans* blocks activation and conversion of complement C3

   Shanshan Luo, Andrea Hartmann, Hans-Martin Dahse, Christine Skerka, and Peter F. Zipfel. *J Immunol*. Jul 19, 2010 (in press).

   Major aspects of the manuscript

   In this manuscript, Pra1, which is secreted by human pathogenic yeast *C. albicans*, was identified as the first fungal complement inhibitor. Pra1 by complexing C3 in solution blocks the conversion of this central complement component by C3 convertases. Thereby, Pra1 inhibits further complement activation, amplification and progression, and down-stream complement effector functions, such as C3a and C5a mediated inflammatory t and C3b/iC3b mediated phagocytosis.

   Own contribution and contribution of the coauthors to the manuscript

   Shanshan Luo has planed, performed, and interpreted the following experiments: ELISA analysis of *Candida* Pra1 and *S. aureus* Efb binding to C3 and C3b; preparation of Factor H depleted human serum; hemolytic assay, Western blotting and C5a ELISA analysis of Pra1 inhibiting on complement activation both in NHS and in Factor H depleted human serum; the mechanism of Pra1 mediated inhibition on complement activation; effect of Pra1 on C3a generation, flow cytometry analysis of C3b/iC3b surface deposition and phagocytosis assay; evaluation of native Pra1 concentration in the culture supernatant of *C. albicans*. Shanshan Luo also wrote the manuscript.

   - Andrea Hartmann has performed the differentiation of the extra- and intra- cellular yeast cells which interact with human macrophages by confocal microscopy.
   - Hans-Martin Dahse assisted in the cell culture and phagocytosis assay by flow cytometry.
   - Christine Skerka discussed the results and wrote the manuscript.
   - Peter F. Zipfel designed the study, interpreted the results and wrote the manuscript.

3. The pH-regulated antigen 1 of *Candida albicans* interacts with C4b-binding protein (C4BP) and mediates fungal contact with human endothelial cells

   Shanshan Luo, Anna M. Blom, Steffen Rupp, Bernhard Hube, Uta-Christina Hipler, Christine Skerka, Peter F. Zipfel.

   Manuscript in revision at the Journal of Biological Chemistry, 2010

   Major aspects of the manuscript

   In this manuscript, I identified Pra1 as the first fungal C4BP binding protein from *C. albicans*. C4BP bound to Pra1 via SCR4, SCR7 and SCR8 maintains cofactor activity. A Pra1 overexpressing strain binds twice C4BP, and Pra1 knock out mutant showes reduced C4BP binding, compared to the wild type strain. Upon co-cultivation of *C. albicans* with human cells, Pra1 surface expression level is up-regulated. By binding to HUVEC cells, surface Pra1 mediates *C. albicans* infection to human endothelial cells. A homozygous nucleotide exchange (A73G) in *PRA1* gene that causes an Asn25Asp exchange on the protein level was identified in all tested strains.

   Own contribution and contribution of the coauthors to the manuscript

- Shanshan Luo has planed, performed and interpreted the following experiments: ELISA analysis of Pra1binding to C4BP, competition of Factor H and C4BP binding to Pra1, and effect of NaCl on Pra1-C4BP interaction; localization of Pra1 binding domains within C4BP; cofactor assay; flow cytometry and confocal microscopy analysis of binding of C4BP to a Pra1 overexpressing , a Pra1 knock out and a wild type *C. albicans* strains, and binding of Pra1 to human cells; flow cytometry analysis of Pra1 expression level at stimulated *C. albicans* surface; adhesion and invasion assay; analysis of Pra1 surface expression and sequence variation in different clinical isolates; Shanshan Luo also wrote the manuscript.
- Anna M. Blom provided the recombinant C4BP, the C4BP deletion mutants, the monoclonal antibody (MAb) 67, MAb 104 and polyclonal rabbit C4BP anti-serum, discussed the results and wrote the manuscript.
- Steffen Rupp had provided a Pra1 overexpressing *C. albicans* strain.
- Bernhard Hube provided a GFP labeled *C. albicans* strain and a Pra1 knock out *C. albicans* strain.
- Uta-Christina Hipler provided clinical *C. albicans* isolates.

- Christine Skerka discussed the results and wrote the manuscript.
- Peter F. Zipfel designed the study, interpreted the results and wrote the manuscript.

4. Complement regulator Factor H mediates a two-step uptake of *Streptococcus pneumoniae* by human cells.

   Vaibhav Agarwal, Tauseef M. Asmat, **Shanshan Luo,** Inga Jensch, Peter F. Zipfel, and Sven Hammerschmidt

   J Biol Chem. 2010 Jul 23; 285(30):23486-95.

   Main points of the manuscript

   In this study, the way how bacterial-bound Factor H promotes pneumococcal uptake and phagocytosis by human cells was identified. Anti-CD11b and anti-CD18 reduce Factor H-promoted uptake by CR3 expressing epithelial cells and PMNs. As a CR3 binding protein, *Candida* Pra1 blocks Factor H-promoted pneumococcal uptake by lung epithelial cells as well as phagocytosis by PMNs, but does not affect adherence. Invasion of pneumococci via Factor H requires the dynamics of host-cell actin microfilaments. Pneumococcal uptake depends on protein tyrosine kinases and phosphatidylinositol-kinase (PI3K).

   Own contribution and contribution of the coauthors to the manuscript

- Shanshan Luo has planed, performed and interpreted recombinant Pra1 expression and purification by FPLC and characterization of polyclonal Pra1 anti-serum.
- Vaibhav Agarwal has planed, performed and interpreted the following experiments: Pneumococcal host cell adherence and invasion assay; Flow cytometric analysis of Factor H binding to pneumococci; fluorescence microscopy assay for Pneumococci attached to host epithelial cells. Vaibhav Agarwal also contributed to the writing of the manuscript.
- Peter F Zipfel provided monoclonal antibody against SCRs19-20, discussed the results and wrote the manuscript.
- Sven Hammerschmidt designed the study and wrote the manuscript.

4. Manuscripts

4.1 Immune evasion of the human pathogenic yeast *Candida albicans*: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein.

Shanshan Luo, Sophia Poltermann, Anja Kunert, Steffen Rupp, Peter F. Zipfel

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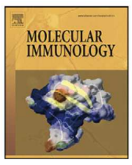

Immune evasion of the human pathogenic yeast *Candida albicans*: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein

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ABSTRACT

The pathogenic yeast *Candida albicans* utilizes human complement regulators, like Factor H and Factor H like protein-1 (FHL-1) for immune evasion. By screening a *C. albicans* cDNA expression library, we identified the pH-regulated antigen 1 (Pra1) as a novel Factor H and FHL-1 binding protein. Consequently Pra1 was recombinantly expressed in *Pichia pastoris* and purified from culture supernatant. Recombinant Pra1 binds Factor H, FHL-1 and also plasminogen. Attached to Pra1, the three human proteins are functionally active. Factor H and FHL-1 inactivate complement and plasminogen can be activated to plasmin which then degrades the extra-cellular matrix component fibrinogen. Polyclonal Pra1 anti-serum was generated and used to localize Pra1 on the surface and also in the culture supernatant of both yeast cells and the hyphal form of *C. albicans*. Furthermore Pra1 expression was up-regulated upon induction of hyphal growth. Pra1, released by *Candida* cells binds back to the surface of Candida hyphae and in addition enhances the complement regulatory activity of Factor H in the fluid phase. A Pra1 overexpression strain, with about twofold higher levels of Pra1 on the surface binds more Factor H, and plasminogen. In summary, *C. albicans* Pra1 is a yeast immune evasion protein that binds host immune regulators and acts at different sites. As a surface protein, Pra1 acquires the two human complement regulators Factor H, FHL-1 and plasminogen, mediates complement evasion, as well as extra-cellular matrix interaction and/or degradation. As a released protein, Pra1 enhances complement control in direct vicinity of the yeast and thus generates an additional protective layer which controls host complement attack.

1. Introduction

In the last years, fungal infections have become a major economic and important health problem (de Berker, 2009; Richardson, 2005). *Candida albicans* is the most frequently fungal pathogen isolated from infected individuals and infections with Candida can range from superficial to systemic disorders (Tuomanen, 1996). Despite currently applied anti-fungal therapies, both mortality and morbidity mediated by human pathogens are still unacceptably high (Alonso-Valle et al., 2003; Gudlaugsson et al., 2003; Pappas et al., 2003). Therefore, new prophylactic and therapeutic strategies are urgently needed to prevent fungal infection. The identification of novel targets in form of yeast virulence factors that contribute to pathogenicity is necessary in defining new strategies to fight and interfere with *Candida* infections.

The complement system, which is a central part of the host innate immune defense forms a major and immediately acting barrier for invading microbes and pathogens (Walport, 2001a; Zipfel and Skerka, 2009). Complement is activated by three pathways which differ considerably in the initial steps. The alternative pathway (AP) which is initiated spontaneously and constantly generates C3b molecules which bind directly to any surface. The lectin pathway (LP) is activated upon binding of mannose-binding lectin to mannan-containing structures on microbial surfaces. The classical pathway (CP) is activated via antigen–antibody complexes. All three pathways activate C3 as the central component of the complement cascade, generating C3a and C3b (Walport, 2001a,b; Zipfel et al., 2007a). The cleavage product C3b, binds to microbial surfaces where it acts as opsonin and mediates recognition by host immune effector cells for phagocytosis (van Lookeren Campagne et al., 2007).

On the surface of host cells complement activation is controlled by multiple regulators which are distributed either in the fluid phase or on the surface. Factor H is the major fluid-phase complement regulator that controls alternative pathway activation at the level of C3. The 150-kDa Factor H protein is exclusively composed of 20 repetitive protein domains, termed short consensus repeats

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(SCRs) (Rodriguez de Cordoba et al., 2004). Factor H is a member of a protein family, which includes the Factor H like protein-1 (FHL-1) which is derived from an alternatively spliced transcript of the Factor H gene, and five Factor H related proteins (CFHRs) that are encoded by separate genes (Zipfel et al., 1999). Factor H and FHL-1 control complement activation by acting as cofactors for the serine protease Factor I, which cleaves C3b into iC3b. In addition, Factor H and FHL-1 compete with Factor B for C3b binding and accelerate the decay of a preformed alternative pathway C3 convertase. Thus, both Factor H and FHL-1 regulate the amplification of complement activation via the alternative pathway by dissociation of C3b from complexes and by displaying cofactor activity in the conversion of C3b to haemolytically inactive degradation products by factor I. Both proteins act in fluid phase in plasma and also on the surface of cells and pathogens (Zipfel et al., 1999; Whaley and Ruddy, 1976).

In order to survive and to establish an infection, pathogens need to inhibit the host complement attack. Apparently pathogens utilize multiple and distinct escape strategies. Human pathogenic fungi, like *C. albicans* and *Aspergillus fumigatus* acquire complement regulators Factor H, FHL-1 and C4BP from human plasma to their surface (Zipfel et al., 2007b; Meri et al., 2002, 2004; Behnsen et al., 2008). Bound to the surface, these human regulators retain complement regulatory functions and inhibit complement activation. Thus, acquisition of human regulators can mask the fungal surface and consequently inhibits and prevents immune attack (Meri et al., 2002, 2004). Furthermore, phosphoglycerate mutase (Gpm1), which is also termed *Candida* CRASP1 (Complement regulator acquiring surface protein) is the first fungal Factor H, FHL-1 binding surface protein identified from *C. albicans* (Poltermann et al., 2007).

Numerous pathogens bind Factor H, FHL-1 and C4BP and utilize these surface bound complement regulators for complement evasion. Such pathogens include Gram-negative bacteria, *Borrelia burgdorferi* (Alitalo et al., 2001; Kraiczy et al., 2001), *Pseudomonas aeruginosa* (Kunert et al., 2007), *Neisseria meningitidis* (Ram et al., 1999), Gram-positive bacteria, like *Streptococcus pyogenes* (Johnsson et al., 1998; Kotarsky et al., 1998), *Staphylococcus aureus* (Haupt et al., 2008), parasites such as *Onchocerca volvulus*, *Echinococcus granulosus* (Diaz et al., 1997), and human viruses like the immunodeficiency *virus* and *West nile virus* (Stoiber et al., 1997; Diamond et al., 2009). For some of these pathogens, the binding proteins for the human regulators have been identified, such as CRASPs of *B. burgdorferi* (Kraiczy et al., 2003; Hartmann et al., 2006; Cordes et al., 2006), Tuf of *P. aeruginosa* (Kunert et al., 2007), the M protein of *S. pyogenes* (Blackmore et al., 1998) and Sbi of *S. aureus* (Haupt et al., 2008).

The pH-regulated antigen 1 (Pra1) of *C. albicans* is composed of 299 amino acids and has a predicted molecular mass of 31 kDa (Sentandreu et al., 1998). Pra1 was originally identified as a fibrinogen binding protein (Casanova et al., 1992). Here we identify *Candida* Pra1 as a new Factor H, FHL-1 and plasminogen binding protein which functions at different sites. As a surface protein, Pra1 acquires the human proteins onto the yeast surface, masks the fungal surface and therefore aids in immune and complement evasion, as well as the degradation of the extra-cellular matrices. Pra1 is also released into the fluid phase where Pra1 enhances Factor H-mediated complement inactivation in direct surrounding of yeast cells.

2. Materials and methods

2.1. C. albicans strains and growth conditions

The *C. albicans* wild type strains SC5314 (Fonzi and Irwin, 1993) CA14 and a Pra1 overexpression strain on a CA14 background (manuscript in preparation) were cultivated in YPD medium (2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract) at 30 °C. Hyphal growth was induced in RPMI 1640 liquid medium (BioWhittaker, Lonza) by temperature shift from 30 to 37 °C for 1.5 h. Yeast cells were collected by centrifugation and counted with a hemocytometer (Fein-Optik, Bad Blankenburg, Germany).

2.2. Antibodies and proteins

Polyclonal Pra1 anti-serum was raised by immunizing rabbits with purified recombinant Pra1. Alexa Fluor®-488 or 647 labeled goat anti-rabbit, Alexa Fluor®-647 labeled rabbit anti-goat and Alexa Fluor®-488 labeled rabbit anti-mouse (Molecular Probes) were used as secondary anti-sera for flow cytometry or confocal microscopy. Anti-sera against goat-, mouse-, and rabbit IgG that were raised in rabbits or swine (pigs) and conjugated with horseradish peroxidase-conjugated were obtained from Dako (Glostrup, Denmark). A monoclonal mouse His antibody was purchased from Qiagen (Hilden, Germany). Polyclonal goat anti-Factor H (Calbiochem) and a polyclonal goat anti-plasminogen (Acris, Hiddenhausen, Germany) were used for assaying Factor H and plasminogen binding. Factor H, Factor I and C3b were obtained from Calbiochem, uPA was purchased from Chemicon (Hofheim, Germany), plasminogen from Chromogenix (Milano, Italy) and BSA was obtained from Sigma.

2.3. Screening of cDNA library

A cDNA (λgt11) library generated from *C. albicans* cDNA was plated and screened for Factor H binding proteins (Cha et al., 1997). Two positive clones were identified and isolated. The inserts were amplified by PCR, the nucleotide sequence was determined.

2.4. Expression and purification of recombinant proteins

The *C. albicans PRA1* gene was amplified by PCR using genomic DNA from strain SC5314 and primers S1 (5′-GGGAATTCGGATGAATTATTTATTGTTTTGTT-3′) and S2 (5′-CGTCTAGAATACAGTGGACTCACCATCTGCA-3′). EcoRI and XbaI restriction sites are underlined. The resulting DNA fragment contained the complete *CaPRA1* coding region which was flanked by EcoRI and XbaI restriction sites. Following restriction digest, this DNA fragment was sub-cloned into *Escherichia coli* cloning vector pCR4Blunt-TOPO (Invitrogen), amplified, isolated and subsequently cloned into the EcoRI and XbaI sites of the *Pichia pastoris* vector pPICZαB (Invitrogen). Pra1 was recombinantly expressed as a His-tagged protein in *P. pastoris* strain X33. Protein expression was induced with 1% methanol. After 3 days of expression, the culture supernatant was harvested.

FHL-1 (SCRs 1–7) and recombinant deletion constructs of Factor H (SCRs 1–5, SCRs 1–6, SCRs 8–11, SCRs 11–15, SCRs 15–18 and SCRs 19–20) were expressed in the baculovirus system as described (Kuhn and Zipfel, 1995). All recombinant proteins were purified by nickel affinity chromatography using HisTrap columns in an Äkta FPLC system (GE Healthcare, Freiburg, Germany) and concentrated using Centricon® Plus-20 concentrators with a cut off of 10 kDa (Millipore).

2.5. ELISA

Pra1 or various Factor H deletion mutants (0.5 μg in carbonate-bicarbonate buffer) was immobilized onto a microtiter plate (MaxiSorb, Nunc) at 4 °C overnight. After washing, nonspecific binding sites were blocked with DPBS containing 1% BSA (Sigma) for 2 h at room temperature (RT). Then Factor H, FHL-1, plasminogen or Pra1 was added (1 μg/well) and the mixture was incubated for 1.5 h at RT. Wells were washed with DPBS-T buffer (DPBS contain-

ing 0.05% Tween 20) and primary anti-serum was added for 1 h at RT. After washing with DPBS-T, horseradish peroxidase-conjugated secondary anti-serum was added and incubated for 1 h at RT. Bound proteins were detected with substrate OPD (o-phenylenediamine dihydrochloride, Sigma). After stopping with 2 M H_2SO_4, the optical density was measured at 492 nm in an ELISA plate reader (SpektraMax 190, Molecular Devices).

2.6. Ligand affinity blotting

Factor H was separated by SDS-PAGE and transferred to a membrane as described (Hartmann et al., 2006). Membranes were blocked with 1× Roti-block (Roth) supplemented with 2.5% BSA in PBS overnight at 4 °C and incubated with recombinant Pra1. After intensive washing specific Pra1 anti-serum was added and bound protein was visualized by ECL.

2.7. Candida ELISA and inhibition assay

Binding of recombinant Pra1 to intact *C. albicans* was measured using a *Candid*a ELISA (Poltermann et al., 2007; Kunert et al., 2007). Briefly, *C. albicans* yeast or hyphae cells were washed with DPBS, diluted into carbonate-bicarbonate buffer to a concentration of 1×10^7 cells/ml and immobilized onto a microtiter plate (MaxiSorb, Nunc) at 4 °C overnight. Wells were washed and nonspecific binding sites were blocked. Following washing, yeast cells or hyphae were incubated with recombinant Pra1 for 1.5 h at 37 °C. For inhibition experiments, immobilized *C. albicans* were pretreated by different dilutions of specific Pra1 anti-serum or pre-immune serum for 2 h at RT. Following extend washing by DPBS, Factor H, FHL-1 or plasminogen was added and incubated for 1.5 h. After washing with DPBS-T (DPBS containing 0.05% Tween 20), the primary antibody was added to the cells for 1 h at RT, followed by addition of a horseradish peroxidase-conjugated secondary anti-serum for another 1 h at RT. Then the bound proteins were detected after additional of the substrate OPD. The reaction was stopped by 2 M H_2SO_4 and the optical density was measured at 492 nm in an ELISA plate reader.

2.8. Cofactor assay

Cofactor activity of the complement regulators Factor H and FHL-1 bound to Pra1 was assayed as described (Meri et al., 2002). Briefly, Pra1 was coated onto the surface of a microtiter plate (MaxiSorb; Nunc) overnight at 4 °C. After washing, nonspecific binding sites were blocked and Factor H or FHL-1 was added (each at 0.4 μg/well). Following extensive washing with DPBS, C3b (0.4 μg/well) together with Factor I (0.8 μg/well) was added. The mixture was incubated for 15 min at 37 °C and stopped by addition of reducing buffer. Fluid-phase cofactor activity was assayed by adding recombinant Pra1 to Factor H, Factor I and C3b, then incubated for 30 min at 37 °C. The reaction mixtures were separated by SDS-PAGE, transferred to a membrane and C3b degradation products were visualized by Western blotting using a polyclonal goat C3 anti-serum (Calbiochem) together with a secondary horseradish peroxidase-conjugated goat anti-serum.

2.9. Activation of plasminogen and assaying plasmin activity

The proteolytic activity of activated plasmin was measured as described (Poltermann et al., 2007). Briefly, Pra1 (0.1–0.5 μg) was immobilized onto a microtiter plate (MaxiSorb, Nunc). After blocking with DPBS containing 1% BSA, plasminogen (1.2 μg/well) was added for 2 h at RT. Unbound plasminogen was removed by washing with DPBS/substrate buffer (0.32 M Tris–HCl, 1.77 M NaCl, pH 7.5). Plasminogen activator uPA (4 ng/well) and chromogenic substrate S-2251 (D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride dissolved in substrate buffer, 150 μg/well; Sigma) were added. Plasmin activity was determined by recording the absorbance at 405 nm (SpektraMax 190, Molecular Devices) in 30 min intervals for 48 h at 37 °C.

In addition, the degradation of native substrate fibrinogen by activated plasmin was assayed. Pra1 bound plasminogen were prepared as above. Fibrinogen (0.5 μg, plasminogen-depleted; Calbiochem) together with plasminogen activator uPA (4 ng/well) was added and the mixture was incubated at RT. At the indicated time points (0, 1, 2 and 4 h), the reaction was stopped by the addition of reducing buffer. The sample was heated for 5 min at 95 °C, separated by SDS-PAGE and transferred to a membrane, the appearance of the degradation products were monitored by Western blotting using a polyclonal fibrinogen anti-serum (Calbiochem) and a secondary HRP-conjugated anti-rabbit serum.

2.10. Flow cytometry and immunofluorescence assays

To verify surface expression of Pra1 on *C. albicans*, yeast and hyphae of wild type strain SC5314, CA14 and Pra1 over expression strain were incubated with rabbit anti-Pra1 or pre-immune serum (1:200 dilution) for 30 min on ice. After washing with blocking buffer (1% BSA in DPBS), an Alexa Fluor®-488 or 647 labeled goat anti-rabbit was added (1:400) and incubated for 30 min on ice. To compare binding of Factor H and plasminogen from normal human serum, CA14 and Pra1 over expression strain were incubated with EDTA-NHS (1:3) or buffer for 1 h at RT. After washing, polyclonal goat anti-Factor H and polyclonal goat anti-plasminogen were added for 30 min on ice, followed by an Alexa Fluor®-647 labeled rabbit anti-goat as a secondary anti-serum. To determine whether Pra1 binds back to the surface of *C. albicans,* yeast cells or hyphae were incubated with recombinant Pra1 (10 μg/10^8) or buffer (negative control) at 37 °C for 1 h. After extensively washing, a Penta-His antibody (1:200) was added for 30 min on ice, followed by an Alexa Fluor®-488 labeled secondary rabbit anti-mouse (1:400). After incubation, samples were washed and measured by flow cytometry (LSR II, BD Biosciences) or confocal microscopy (LSM 510 META, Zeiss, Jena). Forward and sideward scatters were used for the identification of cells, and fluorescent events of 10,000 cells were counted.

3. Results

3.1. Identification of Pra1 as a Factor H binding protein from C. albicans

The pathogenic yeast *C. albicans* binds human complement regulators Factor H and FHL-1 (Meri et al., 2002). In order to identify Factor H binding yeast proteins, a λgt11 cDNA expression library prepared from *C. albicans* cDNA was screened. Two positive clones were identified and isolated. The inserts were amplified by PCR and the nucleotide sequence was determined and corresponded to the pH-regulated antigen 1 (Pra1) (Sentandreu et al., 1998; Lopez-Ribot et al., 1997).

To confirm that *C. albicans* Pra1 is a Factor H binding protein, the *PRA1* gene was amplified and the corresponding insert was sub-cloned into a *P. pastoris* expression vector. Following recombinant expression the His-tagged protein was purified from the culture medium by nickel affinity chromatography. The culture supernatant, flow-through, wash and elute fractions were separated by SDS-PAGE and analyzed by silver staining or by Western blotting. A prominent band of 58 kDa and a band of 38 kDa band were detected in the elute fraction upon silver staining (Fig. 1A, *lane* 4). The 58 kDa band represents Pra1 as confirmed by mass

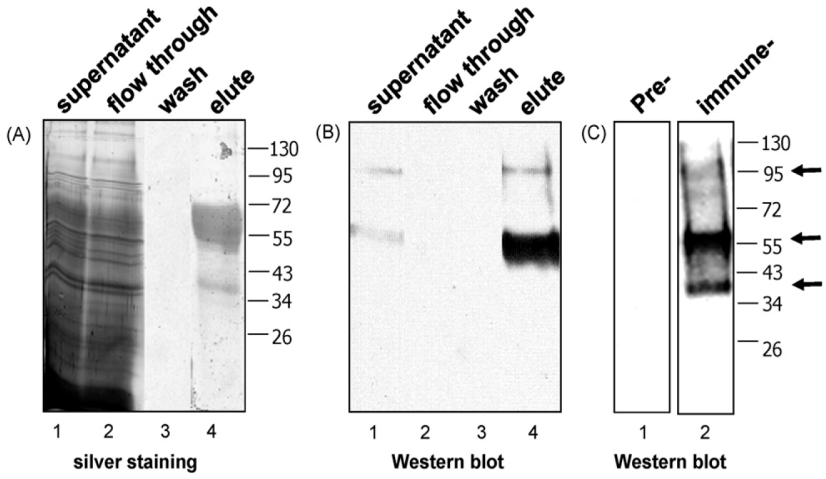

Fig. 1. Recombinant expression of Pra1 and generation of Pra1 specific anti-serum. Pra1 was amplified from *C. albicans* genomic DNA by PCR, cloned into a *Pichia* expression vector and recombinantly expressed in *P. pastoris* as a secreted protein with a C-terminal His-tag. Recombinant Pra1 was purified by affinity chromatography. The culture supernatant, flow-through, wash and elute fractions were separated by SDS-PAGE and analyzed by silver staining and by Western blotting. (A) Silver staining. The *P. pastoris* culture supernatant (*lane* 1), flow-through (*lane* 2), wash (*lane* 3) and elute fractions (*lane* 4) were separated by SDS-PAGE and analyzed by silver staining. (B) Western blotting. *P. pastoris* culture supernatant (*lane* 1), flow-through (*lane* 2), wash (*lane* 3) and elute fractions (*lane* 4) were separated by SDS-PAGE, transferred to a membrane and analyzed by Western blotting using the His anti-serum. (C) Generation of the Pra1 specific anti-serum. Purified Pra1 was used for immunization of rabbits. Reactivity of the Pra1 anti-serum was tested by Western blotting. Pre-immune serum was used as a negative control (*lane* 1), immune serum (anti-Pra1) reacts with recombinant Pra1 (*lane* 2).

spectrometry (data not shown). Western blotting analysis with a His anti-serum identified the 58 kDa band and an additional 95 kDa band in both the culture supernatant and the elute fraction (Fig. 1B, *lanes* 1 and 4). The 58 kDa band represents recombinant Pra1 and the 95 kDa band is most likely Pra1 complexed with other proteins or a dimeric form of Pra1.

Purified Pra1 was used to immunize rabbits and generate Pra1 anti-serum. The immune-, but not the pre-immune serum identified bands with mobilities of 95, 58 and also 38 kDa (Fig. 1C, *lane* 2). Based on the reactivity with Pra1 immune serum and the lack reactivity with the Penta-His anti-serum, the 38 kDa band is considered a degradation product, which has the C-terminal His-tag cleaved off.

3.2. Recombinant Pra1 binds Factor H and FHL-1

To verify that *Candida* Pra1 is Factor H and FHL-1 binding yeast protein, Pra1 was immobilized and binding of purified Factor H and FHL-1 was analyzed. Both complement regulators bound Pra1 (Fig. 2A). Pra1 binding to Factor H was further confirmed by ligand affinity blotting using a reverse setting. Factor H was separated by SDS-PAGE, blotted onto a membrane, incubated with Pra1 and bound Pra1 was detected with the Pra1 specific anti-serum (Fig. 2B, *lane* 1).

To localize the binding domains for Pra1 within Factor H and FHL-1, various deletion mutants were immobilized and binding of Pra1 was analyzed. Pra1 bound to the N-terminal constructs of Factor H and FHL-1, i.e. SCRs 1–5, SCRs 1–6, SCRs 1–7, but not to the deletion mutants representing SCRs 1–4. Similarly Pra1 also bound to C-terminal Factor H constructs, i.e. SCRs 15–18 and SCRs 19–20, but neither to SCRs 8–11 nor to SCRs 11–15 (Fig. 2C). Thus, Pra1 binds the human complement regulator Factor H via two contact sites, which are located within SCRs 5–7 and SCRs 16–20 and binds FHL-1 via one contact site which is located within SCRs 5–7 (Fig. 2E).

In order to characterize the nature of the Pra1–Factor H interaction, the effect of NaCl was analyzed. Pra1 was immobilized and binding of Factor H was assayed upon increasing NaCl concentration. Binding was prominent at low salt conditions, was reduced at physiological concentration (150 mM) and was not detectable at higher NaCl concentration (Fig. 2D). Thus Pra1–Factor H interaction is NaCl dependent and of ionic nature.

3.3. Recombinant Pra1 binds plasminogen

Several Factor H binding proteins of pathogens also bind plasminogen (Poltermann et al., 2007; Kunert et al., 2007; Grosskinsky et al., 2009). We therefore tested if *Candida* Pra1 also binds plasminogen. To this end, Pra1 was immobilized and assayed for plasminogen binding. Plasminogen bound to recombinant Pra1 in a dose dependent manner (Fig. 3A). This interaction was confirmed by slot blot assays using immobilized Pra1 and fluid-phase plasminogen (data not shown). In addition, serum derived native plasminogen did also bind to immobilized Pra1 (Fig. 3B).

The relevance of lysine residues for the Pra1–plasminogen interaction was assayed using the lysine analogue aminocaproic acid (εACA). εACA inhibited plasminogen binding to Pra1 in a dose-dependent manner, reaching about 50% inhibition at a concentration of 0.2 mM and maximal inhibition (100%) at 10 mM (Fig. 3C).

To further characterize the nature of the Pra1–plasminogen interaction, the effect of NaCl was tested. At low concentrations the interaction was prominent and was slightly decreased at the physiological concentration (150 mM) by ca. 20%. Even at a high salt concentration of 600 mM, plasminogen still bound to Pra1 (Fig. 3D). Thus, Pra1–plasminogn interaction is to some extent of ionic nature, however different from that of Factor H.

3.4. Factor H and FHL-1 bound to Pra1 display complement regulatory activity

To define whether Pra1 bound Factor H and FHL-1 maintain cofactor activity, the function of the human regulators attached to Pra1 was assayed. Factor H and FHL-1 were bound to immobilized Pra1, then C3b together with Factor I was added. Cofactor activity of the Pra1 attached regulators is shown by the appearance of C3b cleavage products, α′68, α′46 and α′43 kDa (Fig. 4, *lanes* 1 and 2). No cleavage was observed when BSA was immobilized or in the absence of Factor I (Fig. 4, *lanes* 3–6). Thus, Factor H and

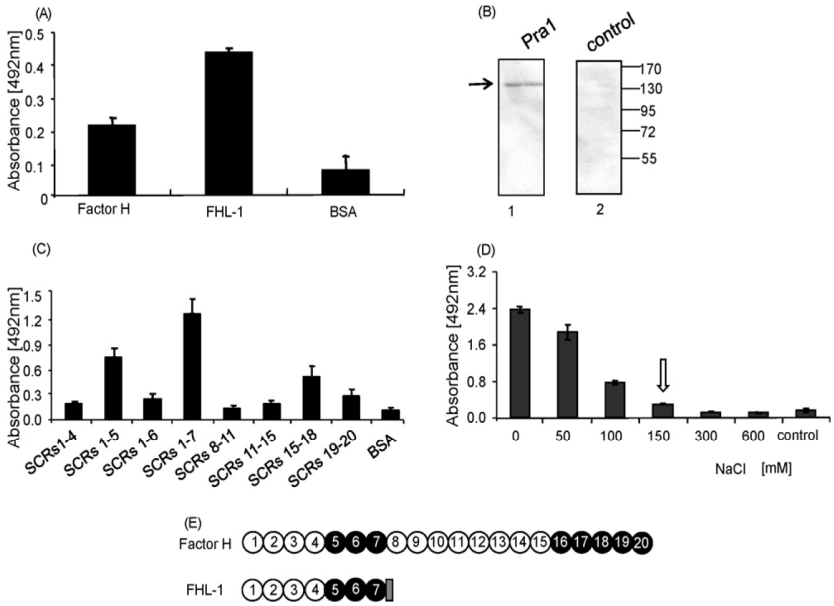

Fig. 2. *C. albicans* Pra1 binds Factor H and FHL-1. (A) Factor H and FHL-1 bind Pra1 as assayed by ELISA. Pra1 was immobilized, Factor H and FHL-1 were added. Both complement regulators bind Pra1. (B) Binding of Pra1 to Factor H assayed by ligand affinity blotting. Factor H was separated by SDS-PAGE and blotted onto a membrane, then incubated with recombinant Pra1 and bound Pra1 was detected using specific Pra1 anti-serum. (C) Localization of the binding regions in Factor H that bind to Pra1. Various deletion mutants of Factor H and FHL-1 were immobilized and Pra1 was added. Pra1 bound the N-terminal constructs SCRs 1–5, SCRs 1–6 and SCRs 1–7, but not deletion mutants representing SCRs 1–4. Similarly Pra1 bound C-terminal constructs SCRs 15–18 and SCRs 19–20, but not SCRs 8–11 and SCRs 11–15. (D) Effect of NaCl on Pra1–Factor H interaction as analyzed by ELISA. Pra1 was immobilized, Factor H was added in the presence or absence of indicated concentration of NaCl. At low salt concentrations, binding was prominent and was reduced at the physiological NaCl concentration (150 mM). At higher salt concentrations (>150 mM), binding was almost abolished. (E) Localization of the two Pra1 binding regions in Factor H protein and the single binding region within FHL-1. Pra1 binding regions are highlighted in *black*. The *bars* represent the means of three independent experiments ± SD.

FHL-1 bound to Pra1 maintain complement regulatory function and control complement activation.

3.5. Plasminogen bound to Pra1 is functionally active

To analyze whether plasminogen bound to Pra1 can be converted to active plasmin, plasminogen was bound to the immobilized Pra1 and activated by uPA. Plasmin was generated which cleaved the synthetic chromogenic substrate in a time and dose dependent manner (Fig. 5A).

To further characterize the role of Pra1 attached plasminogen, cleavage of a physiological substrate fibrinogen was analyzed. Again plasminogen was bound to immobilized Pra1, activated by uPA and fibrinogen was added. The reaction mixture was sepa-

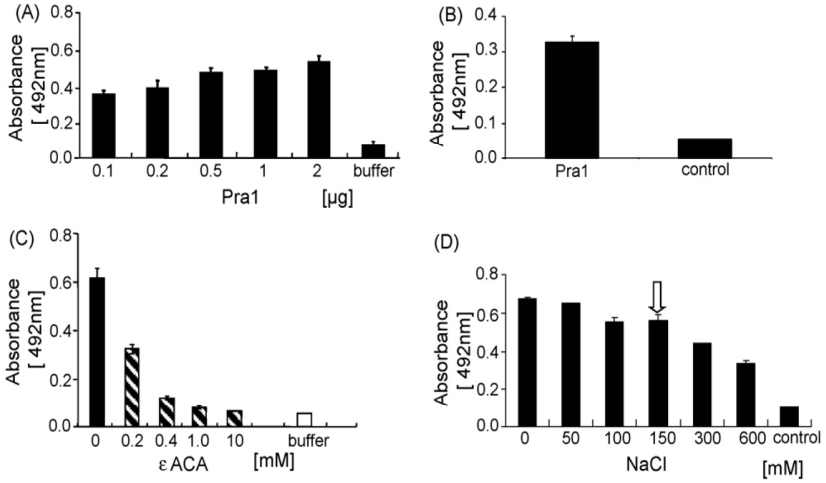

Fig. 3. *Candida* Pra1 binds plasminogen. (A) Binding of purified plasminogen to Pra1 was analyzed by ELISA. The indicated amounts of recombinant Pra1 were immobilized, purified plasminogen was added and plaminogen binding was assayed with a polyclonal goat plasminogen anti-serum. Purified plasminogen binds Pra1 dose dependently. (B) Binding of plasminogen derived from human serum to recombinant Pra1. Pra1 was immobilized, human serum was added, and bound plasminogen was assayed using a polyclonal goat plasminogen anti-serum. (C) Lysine residues mediate plasminogen–Pra1 interaction. Pra1 was immobilized, plasminogen together with the indicated concentrations of εACA was added. Binding of plasminogen to Pra1 was detected by a polyclonal goat anti-plasminogen. εACA inhibits plasminogen binding to Pra1. (D) Effect of NaCl on plasminogen–Pra1 interaction. Pra1was immobilized, plasminogen was added in the presence or absence of indicated concentrations of NaCl. At low NaCl concentrations, binding was strong, and decreased in higher concentrations of NaCl. The *bars* represent the means of three independent experiments ± SD.

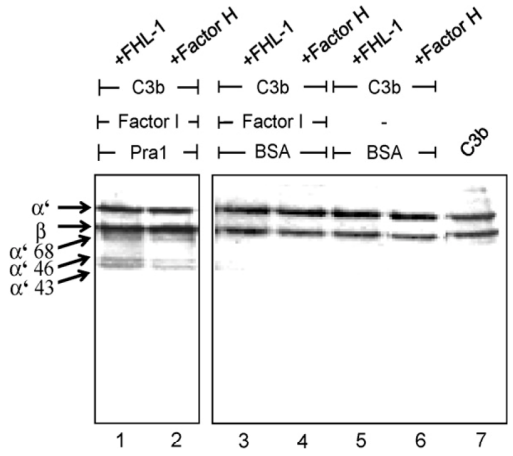

Fig. 4. Factor H and FHL-1 bound to Pra1 retain complement regulatory activity. Pra1 was immobilized, FHL-1 (*lane* 1) or Factor H (*lane* 2) were applied, and after extensive washing, then C3b and Factor I were added. The mixture was incubated for 15 min at 37 °C, the supernatants were separated by SDS-PAGE and transferred to a membrane. C3b cleavage products were detected by a polyclonal C3 anti-serum. C3b degradation was visualized by the appearance of the α′68-, α′46-, and α′43-kDa bands. No cleavage products were detected in the absence of Pra1 or Factor I (*lanes* 3–7). A representative experiment out of five is shown.

rated by SDS-PAGE and fibrinogen degradation was analyzed by Western blotting. Fibrinogen was degraded in a time dependent manner (Fig. 5B, *lanes* 1–4) and after 4 h fibrinogen was completely degraded (Fig. 5B, *lane* 4). Thus, plasminogen bound to Pra1 is accessible for the activator uPA, the activated plasmin is proteolytically active and cleaves both the synthetic and the native substrate.

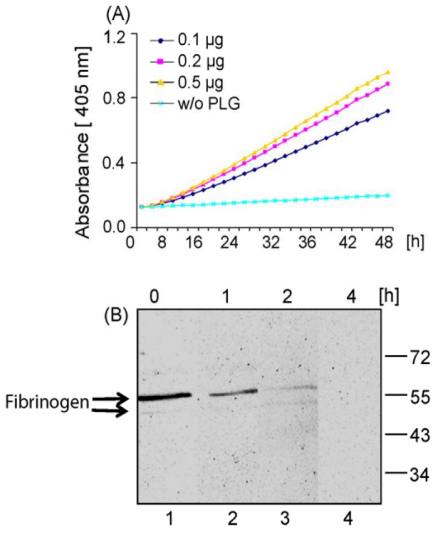

Fig. 5. Plasminogen bound to Pra1 is functionally active. (A) Degradation of chromogenic substrate. Indicated amounts of recombinant Pra1 were immobilized, then plasminogen was added. After extensive washing, the activator uPA and the chromogenic substrate S-2251 were added and the absorbance was measured at the indicated time points. Proteolytic plasmin activity was time and dose dependent. (B) Degradation of fibrinogen. Pra1 was immobilized, then incubated with plasminogen. After extensive washing, the activator uPA and fibrinogen were added. At 0, 1, 2 and 4 h (*lanes* 1–4), the reaction was stopped by adding reducing buffer. Finally all the samples were separated by SDS-PAGE and degradation of fibrinogen was analyzed by Western blotting using a polyclonal rabbit anti-fibrinogen. Fibrinogen degradation was time dependent (*lanes* 1–4). After 4 h incubation, fibrinogen was completely degraded (*lane* 4). The results are representative data of five independent experiments.

3.6. Localization of Pra1 at the surface and as a released protein

Pra1 specific anti-serum was applied to confirm both surface localization and release of the protein. Upon incubation with Pra1 anti-serum yeast cells showed a prominent fluorescence signal as revealed by flow cytometry (Fig. 6A, *blue line*). No signal was detected when yeast cells were incubated with pre-immune serum or buffer. Surface expression of Pra1 on *C. albicans* was also confirmed by confocal microscopy. Pra1 was detected on the surface of both yeast cells and hyphae. Pra1 expression was enhanced on the surface of hyphae and the fluorescence signal was particularly prominent at the tip of the hyphae (Fig. 6B). Thus, Pra1 is present on the surface of both the yeast and hyphal form of *C. albicans* and Pra1 surface expression is stronger upon hyphal induction.

To assay that Pra1 is a released protein, culture supernatant was harvested at different time points, separated by SDS-PAGE, transferred onto a membrane and the presence of Pra1 was assayed by specific anti-serum. The anti-serum identified both a 68 kDa and a 130 kDa band in the culture medium of yeast cells harvested after 24 h (Fig. 6C, *lanes* 2–5). In culture medium derived from hyphae which were treated for 1.5 h with RPMI 1640 medium, bands with the same mobility were detected however at a higher intensity (Fig. 6C, *lanes* 2 and 6).

3.7. Soluble Pra1 binds back to the surface of the hyphal form of *C. albicans*

Pra1 is a surface protein and is also released. Therefore we asked whether soluble Pra1 can bind back to the surface of *C. albicans*. To this end, both *Candida* yeast and hyphal cells were immobilized, recombinant Pra1 was added as a ligand in the fluid phase and bound Pra1 was detected by a His anti-serum. This anti-serum specifically identifies the recombinant-, but not the native Pra1. Recombinant Pra1 was not detectable on the surface of yeast cells (Fig. 7A, *hatched columns*), but was identified on the surface of hyphae. Binding was dose dependent (Fig. 7A, *black columns*). Binding of soluble Pra1 to *C. albicans* was further confirmed by immune fluorescence. Again no binding of recombinant Pra1 to the yeast cells was detected (data not shown), but binding of Pra1 to the hyphal form was revealed by the green fluorescence (Fig. 7B, *left panel*). Thus, Pra1, which is released by *Candida*, binds back to the surface of the hyphal form of *C. albicans*.

3.8. Pra1 enhances cofactor activity of Factor H in fluid phase

Pra1 is released by yeast cells. We therefore ask whether Pra1 may affect complement regulation in the fluid phase. To test this, the effect of Pra1 on Factor H-mediated cofactor activity was analyzed. Factor H, C3b and Factor I were incubated for 30 min at 37 °C in solution in the presence or absence of Pra1. The mixtures were separated by SDS-PAGE, transferred to a membrane and C3b degradation products were identified by Western blotting. Pra1 enhanced cofactor activity of Factor H in a dose dependent manner, as revealed by the disappearance of α′110 kDa band and appearance of the α′46 and α′43 kDa bands (Fig. 8A, *lanes* 2–5). The decrease of α′110 kDa bands was quantified by densitometry and at 5 µg Pra1 enhanced C3b degradation by ca. 60% (Fig. 8B). Thus, released Pra1 enhances complement control and C3b inactivation in solution.

3.9. Native Pra1 at the surface of *C. albicans* acquires Factor H, FHL-1 and plasminogen

To confirm that native surface exposed Pra1 binds Factor H, FHL-1 as well as plasminogen, yeast cells were immobilized and Pra1 on the surface of the yeast cells was blocked with the specific anti-serum. Following incubation of treated yeast cells with each

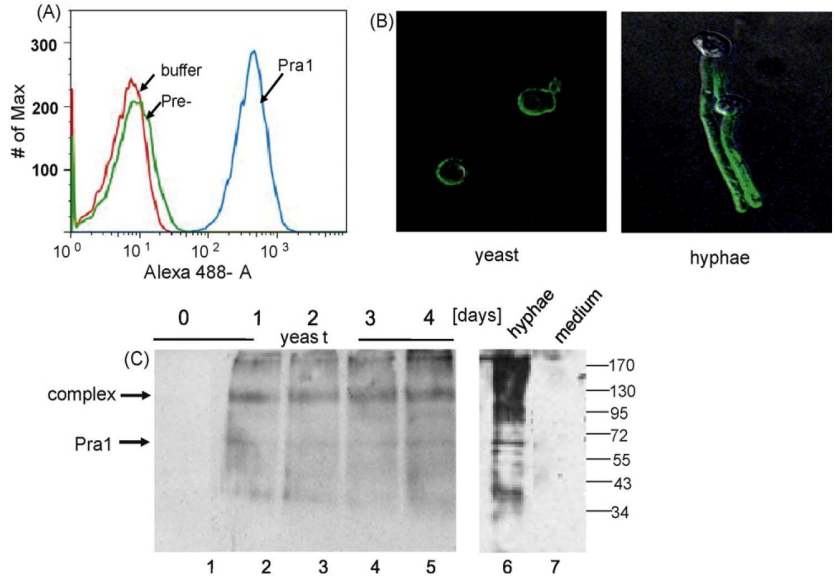

Fig. 6. Localization of Pra1 on live *C. albicans*. (A) Pra1 is a surface protein of yeast *C. albicans* was analyzed by flow cytometry. The yeast form of *C. albicans* was incubated with Pra1 anti-serum for 30 min on ice and then an Alexa Fluor®-488 labeled polyclonal goat anti-rabbit antibody was added. Yeast cells showed a prominent fluorescence signal when incubated with Pra1 anti-serum (*blue line*). No signal was detected when the cells were incubated in DPBS or in pre-immune serum (*red and green lines*). (B) Location of Pra1 by confocal microscopy. Yeast and hyphae of *C. albicans* were incubated with Pra1 anti-serum for 30 min on ice and an Alexa Fluor®-488 labeled polyclonal goat anti-rabbit serum was added. Pra1 is detected at the surface of yeast and hyphal forms of *C. albicans*. For the hyphal form, the fluorescence signal was prominent on the tip. The signal on the surface of hyphae is stronger than that on the yeast. (C) Pra1 is released into the culture medium. *C. albicans* was cultivated in YPD medium and after 0, 1, 2, 3 and 4 days (*lanes* 1–5), the culturing medium was harvested and separated by SDS-Page. Pra1 expression was analyzed by Western blotting using polyclonal Pra1 anti-serum. Pra1 is identified as 130 kDa and a 68 kDa band (*lanes* 2–5). Hyphal growth was induced and Pra1 expression in the culture medium of hyphae was again assayed by Western blotting using specific anti-serum (*lanes* 6–7). The intensity of the 68 and 130 bands in the hyphal culture medium are stronger as compared to the yeast culture medium (*lanes* 2 and 6). The results shown are representative out of three separate experiments.

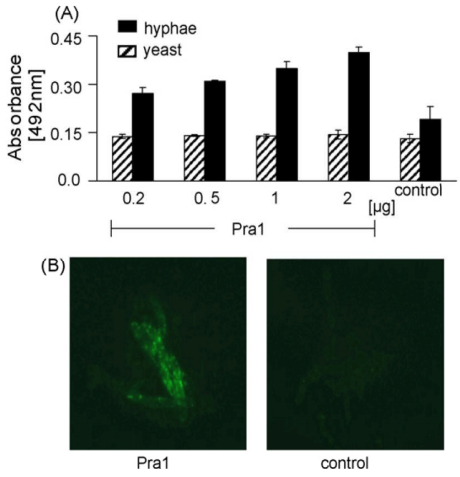

Fig. 7. Pra1 binds back to the surface of *C. albicans* hyphae. (A) Pra1 binds back to the surface of hyphal form of *C. albicans*. Both yeast and hyphal form of *C. albicans* were immobilized and different amounts of recombinant His-tagged Pra1 were added. Bound Pra1 was detected by a His anti-serum in combination with a secondary anti-mouse serum. Binding of recombinant Pra1 to the yeast cells was not detectable (*hatched columns*), but Pra1 bound to hyphal form of *Candida* (*black columns*). The bars represent the mean values of three independent experiments ± SD. (B) Binding of Pra1 to the surface of *C. albicans* was confirmed by immunofluorescence. Hyphal form of *C. albicans* were incubated with recombinant Pra1 at 37 °C for 1 h. Bound Pra1 was detected by a His anti-serum and followed by an Alexa Fluor®-488 labeled monoclonal rabbit anti-mouse serum. Recombinant Pra1 does bind to the *C. albicans* hyphae. A representative experiment out of four independent experiments is shown.

of the three human proteins, binding of Factor H, FHL-1 as well as plasminogen to Candida cells was analyzed. In the presence of the Pra1 anti-serum used at 1:1000 binding of Factor H, FHL-1 or plasminogen to the yeast surface was reduced by ca. 17% (Fig. 9A, *black columns*), 40% (Fig. 9A, *hatched columns*) and 12% (Fig. 9B), respectively. However, pre-immune serum showed no effect. These effects show that Pra1 at the surface of yeast cells binds Factor H, FHL-1 and plasminogen and that in addition to Pra1, other yeast surface proteins exist which bind Factor H, FHL-1 and plasminogen.

To further verify whether Pra1 is relevant for acquisition of Factor H and plasminogen, a Pra1 overexpression strain was analyzed. This Pra1 overexpression strain expressed about twofold more Pra1 on the surface, as compared to the wild type strain (Fig. 9C). Similarly upon incubation of both strains with NHS-EDTA, the Pra1 overexpression strain bound about two times more Factor H and also plasminogen (Fig. 9D). This indicates that surface expressed Pra1 plays a role in *C. albicans* acquiring Factor H and plasminogen from NHS and therefore aids in immune and complement evasion of both yeast and hyphal forms of *C. albicans*.

4. Discussion

Here we identify Pra1 as a new Factor H, FHL-1 as well as plasminogen binding protein from *C. albicans*. Recombinant Pra1 was expressed and purified from *P. pastoris* and used to generate specific anti-serum. Pra1 was localized at the surface and was also identified as a soluble protein released by both yeast and hyphal Candida cells. As a surface protein, Pra1 binds human complement regulators Factor H, FHL-1 as well as plasminogen. The attached human proteins are functionally active and aid in complement evasion or degradation of extra-cellular matrices. As a released protein, *Candida* Pra1 binds back to the surface of hyphal cells and in addition

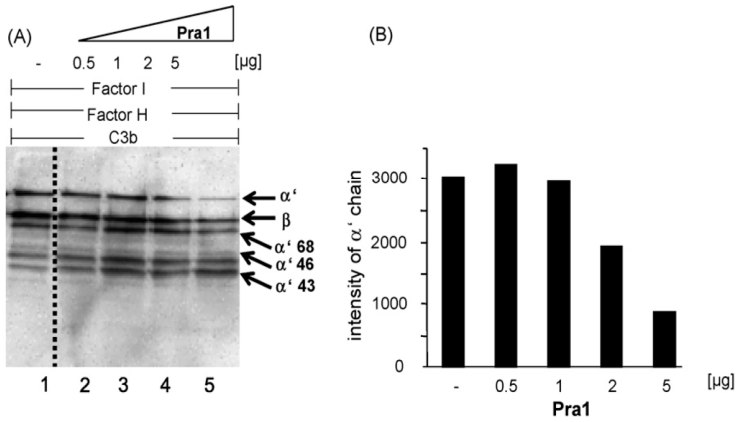

Fig. 8. Pra1 enhances cofactor activity of Factor H in the fluid phase. (A) Cofactor activity of Factor H in fluid phase. Factor H, Factor I and C3b together with indicated amounts of Pra1 were incubated for 30 min at 37 °C. The reaction mixture was separated by SDS-PAGE, transferred to a membrane and C3b and C3b degradation products were identified by Western blotting. Cofactor activity of Factor H is revealed by the disappearance of α′110 kDa and the appearance of cleavage products of α′68, α′46 and α′43 kDa (*lanes* 1–5). The presence of Pra1 enhances cofactor activity of Factor H in a dose dependent manner, as revealed by the disappearance of α′110 kDa band and the appearance of the α′46 and α′43 kDa bands. (B) Densitometry analysis of the α′ chain. The density of α′ chain bands from lanes 1–5 of (A) was determined. Upon increasing the amount of Pra1 the density of the α′ chain decreases in a dose dependent manner. When Pra1 was used at 5 µg, the density was decreased by 60.3%. The data show a representative result of four independent experiments.

enhances complement control in direct vicinity of the pathogen. This action forms an outer layer of immune defense which controls host-mediated complement attack close to the fungal cell wall

Candida Pra1 was initially identified as a fibrinogen binding surface protein (Casanova et al., 1992) and recently Pra1 was identified as a ligand for the human integrin $\alpha_M\beta_2$. Pra1 surface expression is not consistently observed. Cell wall studies suggest that Pra1 is primarily or exclusively localized to hyphal structures depending on the *Candida* strain used (Choi et al., 2003; Chaffin et al., 1998; Tronchin et al., 1987). In addition leukocytes adhere to hyphal filaments but not or rather weakly to the yeast form of *Candida* (Forsyth et al., 1998). Using the specific anti-serum we localized Pra1 at the surface of both yeast- and hyphal Candida cells and also confirmed release of Pra1 into the culture supernatant by both the yeast and

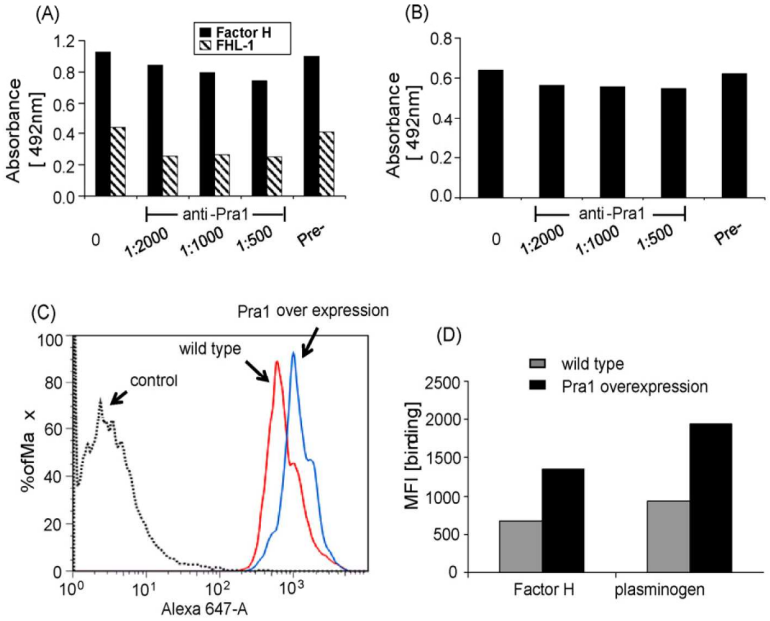

Fig. 9. Native Pra1 at the surface of *C. albicans* acquires Factor H, FHL-1 and plasminogen. (A) Inhibition of Factor H and FHL-1 binding to *C. albicans* by specific Pra1 anti-serum. *C. albicans* yeast cells were immobilized and incubated with the indicated dilutions of Pra1 anti-serum. Then Factor H or FHL-1 was added. Bound Factor H or FHL1 was identified using a polyclonal goat Factor H anti-serum. Pra1 anti-serum inhibits Factor H binding to *C. albicans* in a dose dependent manner. The inhibition for Factor H binding is about 17% (*black columns*), and 40% for FHL-1 (*hatched columns*). (B) Inhibition of plasminogen to *C. albicans* using Pra1 anti-serum. Immobilized *C. albicans* yeast cells were pretreated with the indicated amounts of Pra1 anti-serum, then plasminogen was added. Bound plasminogen was identified using polyclonal goat plasminogen anti-serum. Pra1 anti-serum blocked plasminogen binding to *C. albicans* by ca. 12%. Pre-immune serum showed no inhibitory effect. (C) Pra1 expression level on the surface of both Pra1 overexpression and wild type *C. albicans* strain. Both strains were incubated with polyclonal Pra1 anti-serum, followed by an Alexa Fluor®-647 labeled goat rabbit anti-serum. Pra1 expression level was then analyzed by flow cytometry. The overexpression strain has more Pra1 on the surface. (D) Binding of Factor H and plasminogen to both *C. albicans* strains. The overexpression and the wild type *Candida* strains were incubated in EDTA-NHS, then bound Factor H and plasminogen were detected by flow cytometry using polyclonal goat Factor H or polyclonal goat plasminogen anti-serum. The Pra1 overexpression strain binds about twice more Factor H and plasminogen than the wild type. The results show a representative experiment of four separate experiments.

hyphal forms of *C. albicans*. Surface expression and release were shown by flow cytometry, confocal microscopy and Western blotting (Fig. 6). In *Candida* hyphae Pra1 is localized primarily at the tip of the cells, suggesting an important role of Pra1 upon contact with host tissues and surfaces during infection. Expression of Pra1 is up-regulated upon *C. albicans* switching from the yeast to hyphal growth (Fig. 6), which may also account for the role of Pra1 in *C. albicans* virulence as the hyphal form of *C. albicans* is more invasive than the yeast form (De Bernardis et al., 1998; Muhlschlegel et al., 1998; Sepulveda et al., 1998).

Pra1 is composed of 299 amino acids and has a predicted molecular mass of 31 kDa (Sentandreu et al., 1998). However recombinant Pra1 expressed in *P. pastoris* is identified as a 58- and a 95-kDa protein (Fig. 1). Native Pra1 derived from both the yeast and hyphal form of *C. albicans* has even higher mobilities of 68 and 130 kDa (Fig. 6C). These differences are explained by glycosylation and attachment of carbohydrate side chains (Lopez-Ribot et al., 1997; Chaffin et al., 1998; Polonelli et al., 1994). Pra1 has several consensus sites for N-linked as well as O-linked glycosylation. Treatment of the native protein with endoglycosidase H reduced the molecular mass by ca. 25% (Sentandreu et al., 1998). Based on the reactivity with the polyclonal Pra1 immune serum and the lack reactivity with the His anti-serum, the 38 kDa band is considered a degradation product, which has the C-terminal His-tag cleaved off. Thus, monomeric recombinant Pra1 has a mobility of 58 kDa, as confirmed by mass spectrometry and monomeric native Pra1 an apparent mass of 68 kDa. Consequently the 95 kDa band most likely represents a dimeric form of Pra1.

Pra1 is a new fungal Factor H, FHL-1 and plasminogen binding protein. Pra1 binds the human complement regulator Factor H via two contact sites, which are located within SCRs 5–7 and SCRs 16–20. Pra1 binds FHL-1 via one contact site which is located within SCRs 5–7 (Fig. 2C and E). Binding of Factor H to Pra1 is based on ionic interaction and affected by NaCl (Fig. 2D). Pra1 also binds plasminogen. The inhibition with lysine analogue aminocaproic acid suggests that lysine residues mediate contact between Pra1 and human plasminogen. The Pra1–plasminogen interaction is also based on ionic interaction, however is not as much pronounced as the Pra1–Factor H interaction. Pra1–plasminogen, but not Pra1–Factor H interaction is still detectable at 600 mM NaCl (compare Figs. 3D and 2D).

Attached to Pra1, each human regulator is functionally active. Pra1 bound Factor H and FHL-1 maintain complement regulatory cofactor activity and assist C3b inactivation (Fig. 4). Similarly plasminogen bound to Pra1 is accessible to the activator uPA and is converted into the plasmin. The activated serine protease plasmin degrades both the synthetic chromogenic substrate S-2251 and the native extra-cellular matrix component fibrinogen (Fig. 5). Thus, *C. albicans* yeast and hyphae cells can utilize Pra1 to acquire human regulators onto the surface, to mask the fungal surface from complement attack and also for degradation of the extra-cellular matrices.

Pra1 specific anti-serum inhibits binding of Factor H, FHL-1 and plasminogen to *C. albicans* by ca. 17%, 40% and 12%, respectively (Fig. 9A and B). This inhibitory effect confirms that Pra1 at the yeast surface binds Factor H, FHL-1 as well as plasminogen. This relative low level of reduction is explained by the existence of additional Factor H, FHL-1 as well as plasminogen binding proteins. One additional Candida Factor H/FHL-1 and plasminogen binding protein is the moonlighting protein phosphoglycerate mutase (Gpm1), also termed *Candida* CRASP1 (complement regulator acquiring surface protein) (Poltermann et al., 2007). In addition, eight plasminogen binding proteins have been identified from *C. albicans* by proteome approach, including alcohol dehydrogenase, thioredoxin peroxidase, catalase, transcription elongation factor, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase (Gpm1) and fructose bisphosphate aldolase (Poltermann et al., 2007; Crowe et al., 2003).

Due to the existence of several different Factor H, FHL-1 and plasminogen binding protein on the surface of the yeast cells, a Pra1 knock out mutant will not differ substantially in Factor H, FHL-1 and plasminogen binding. Consequently the Pra1 overexpression strain of *C. albicans* which has increased Pra1 surface expression levels might be more suitable for functional complement escape studies. As expected this Pra1 overexpression strain, which has about twofold more Pra1 levels on the yeast surface, acquires more Factor H and plasminogen from NHS as the wild type strain (Fig. 9C and D) confirming the role of Pra1.

Pra1 is also released into the culture supernatant. Soluble Pra1 aids in complement control and enhances the complement regulatory activity of Factor H for Factor I-mediated cleavage of C3b. Thus released Pra1 enhances complement control in the direct vicinity of *C. albicans* (Fig. 8), and thus provides an additional protective layer which limits and prevents complement attack at the yeast surface. In addition, surface exposed Pra1 binds to the integrin receptor $\alpha_M\beta_2$ expressed on the surface of human leukocytes and may aid in cell interaction (Soloviev et al., 2007). Furthermore, when released by the yeast cells and consequently bound to $\alpha_M\beta_2$ receptor Pra1 may block effector functions and recognition of C3b opsonized yeast cells by human macrophages.

Thus, Pra1 is a multifunctional virulence factor of *C. albicans*, which binds several human immune regulators and acts at different sites. Attached to the surface of *C. albicans*, Pra1 acquires complement regulators Factor H and FHL-1 and modulates complement attack. Pra1 also binds plasminogen which upon activation by uPA facilitates extra-cellular matrix degradation. As a released protein, Pra1 enhances complement control in the surrounding of the pathogen, and thus provides an additional protective defense layer. Released Pra1 also binds back to the surface of the Candida hyphae (Fig. 7A and B) and aids in surface complement control. In addition soluble Pra1 by binding to the integrin receptor $\alpha_M\beta_2$ on human leukocytes may interfere with the effector function and recruitment of phagocytic cells. These multiple effects of Pra1 provide insights into the complex immune escape mechanism used by human pathogenic yeast *C. albicans*. A detailed understanding of the multiple roles of Pra1 allows to define new strategies to interfere with *Candida* infection.

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4.2 Secreted pH-regulated antigen 1 of *Candida albicans* blocks activation and conversion of complement C3.

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Secreted pH-Regulated Antigen 1 of *Candida albicans* Blocks Activation and Conversion of Complement C3

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The complement system forms the first defense line of innate immunity and is activated within seconds upon infection by human pathogenic yeast *Candida albicans*. In this study, we identified a new complement evasion strategy used by *C. albicans*. The fungus secretes a potent complement inhibitor, pH-regulated Ag 1 (Pra1), which in the direct surrounding of the pathogen binds to fluid-phase C3 and blocks cleavage of C3 to C3a and C3b, as shown by ELISA, native gel electrophoresis, and Western blotting. Consequently, complement activation via the alternative and classical pathways is inhibited. In addition, the release of the anaphylatoxins C3a and C5a, as well as C3b/iC3b surface deposition, is reduced, as demonstrated by Western blotting, ELISA, confocal microscopy, and flow cytometry. By reducing C3b/iC3b levels at the yeast surface, Pra1 decreases complement-mediated adhesion, as well as uptake of *C. albicans* by human macrophages, as shown by flow cytometry. Thus, Pra1 is, to our knowledge, the first potent fungal complement inhibitor that favors *C. albicans* immune escape by inactivating and controlling host complement attack at the level of C3. *The Journal of Immunology*, 2010, 185: 000–000.

Candida albicans is an opportunistic human pathogenic fungus that causes life-threatening infections, as well as severe disease, particularly in immunocompromised patients (1). In recent years, the number of resistant *Candida* strains is continuously and rapidly increasing. Despite the currently existing antifungal therapies, mortality and morbidity caused by this human pathogenic fungus are still unacceptably high (2–4). Therefore, it is of general and practical relevance to characterize the immune escape mechanisms of *C. albicans* on a molecular level and to identify novel molecules that mediate fungal infection.

The human complement system is an essential and effective part of innate immunity and, in its normal setting, directly attacks any invading microbe (5–8). Thus, to survive, pathogens have to escape this central immune barrier. The complement system is activated via three major pathways. The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 and is amplified on foreign surfaces that lack complement regulators. The activated system generates C3b molecules that bind to any surface. Surface-deposited C3b binds factor B, which is activated by factor D, and consequently, a C3 convertase of the AP (C3bBb) is generated. The classical pathway (CP) is activated by Ag-Ab complexes or by C-reactive protein, and the lectin pathway (LP) is activated by binding of mannose-binding lectin and ficolins to carbohydrate structures or acetylated compounds on microbial surfaces (6, 9–11). Activation of the CP and LP leads to formation of the C3 convertase (C4b2b).

Upon complement activation, a variety of important immune effector molecules is generated that have important effector functions. For example, after cleavage of C3 and C5, C3a and C5a peptides, which are released into the fluid phase, can recruit immune effector cells to the sites of infection (12, 13). The opsonins C3b/iC3b/C4b are deposited onto surfaces of a particle or microbe, and such targeted particles/microbes are recognized by complement receptors (CR; i.e., CR1, CR3, CR4, and CRIg) that are present at the surfaces of host effector cells. This ligand-receptor interaction results in phagocytosis and efficient removal of opsonized particles or microbes (14, 15).

To survive, pathogens have developed sophisticated mechanisms to inactivate and directly inhibit host complement attack (16). Human pathogenic fungi, such as *C. albicans* and *Aspergillus fumigatus*, acquire soluble human complement regulators (like factor H, factor H-like protein 1 [FHL-1], and C4b-binding protein) to their surfaces. These bound regulators mediate *C. albicans* for complement evasion (17–21). Phosphoglycerate mutase 1 (Gpm1, also termed *Candida* complement regulator acquiring surface protein 1) and pH-regulated Ag 1 (Pra1, or *Candida* complement regulator acquiring surface protein 2) were recently identified as factor H and FHL-1–binding *C. albicans* surface proteins (22, 23). Immune evasion by acquiring human complement regulators (like factor H) is common for many pathogens in addition to human pathogenic fungi, such as Gram-negative bacteria, *Borrelia burgdorferi* (24, 25), *Pseudomonas aeruginosa* (26), *Neisseria meningitidis* (27), and *Haemophilus influenzae* (28); Gram-positive bacteria such as *Streptococcus pyogenes* (29–31) and *Staphylococcus aureus* (32); parasites including *Onchocerca volvulus* (33) and *Echinococcus granulosus* (34); and human viruses like the immunodeficiency virus and West Nile virus (35–37).

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Abbreviations used in this paper: AP, alternative pathway; CP, classical pathway; CR, complement receptor; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; DPBS, Dulbecco's PBS; Efb, extracellular fibrinogen-binding protein; Δfactor B-HS, factor B-depleted human serum; Δfactor H-HS, factor H-depleted, complement active human serum; FB, factor B; FD, factor D; FHL-1, factor H-like protein 1; FP, factor P; HSA, human serum albumin; LP, lectin pathway; MFI, mean fluorescence intensity; NHS, normal human serum; Pra1, pH-regulated Ag 1; RT, room temperature.

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In addition to acquisition of complement regulators, expression of endogenous complement inhibitors is another mechanism used by pathogenic microbes to inactive complement activation. Pathogens like *S. aureus* express extracellular fibrinogen-binding protein (Efb) and its homologous protein (Efb homologous protein) to bind C3, and further inhibits the substrate C3 conversion by the C3 convterases (38, 39) and also expresses Staphylococcal complement inhibitor to bind and stabilize the assembled C3 convertases (40, 41). In addition, *S. aureus* expresses the chemotaxis inhibitory protein to bind and neutralize the C5a receptor on the surfaces of neutrophils and monocytes, thus blocking the anaphylotoxin C5a-mediated chemotaxis (42–44).

Pra1 of *C. albicans* with 299 aa is a glycosylated protein. *PRA1*, *PHR1*, and *PHR2* are three pH-regulated genes of *C. albicans* that are involved in morphogenesis (45). Pra1 is located on the fungal surface and is also secreted into culture supernatant by both yeast and hyphal forms of *C. albicans* (46–48). Pra1, which was originally identified as a fibrinogen-binding protein (46), binds human complement regulator factor H, FHL-1, and plasminogen (23), and is also a ligand for the integrin CR3 (alternative names: $\alpha_M\beta_2$ or CD11b/CD18), which is expressed on the surface of human leukocytes (47).

In this study, we identify secreted *Candida* Pra1 as the first fungal complement inhibitor. Pra1 complexes C3 in solution, thereby blocking C3 activation and conversion by surface-bound C3 convertases of both the AP and CP/LP. Thus, downstream events, such as the complement amplification, generation of the anaphylatoxins C3a and C5a, as well as C3b/iC3b surface deposition are inhibited. This blockade of the human complement response reduces inflammation and C3b/iC3b-mediated adhesion and uptake of yeast by human macrophages.

Materials and Methods

Cultivation of C. albicans *strains and human cell lines*

The *C. albicans* wild-type strain SC5314 (49, 50) was cultivated in yeast–peptone–dextrose medium (2% [w/v] glucose, 2% [w/v] peptone, 1% [w/v] yeast extract) at 30°C. Yeast cells were collected by centrifugation and counted with a hemocytometer (Fein-Optik, Bad Blankenburg, Germany). Human monocytic cells (THP-1) were grown in RPMI 1640 medium, supplemented with 10% FCS, 1% ultraglutamine 1, and 0.055% gentamicin sulfate (Lonza, Verviers, Belgium) at 37°C in 5% CO_2. Macrophages were prepared by stimulating the monocytic cells with PMA (2 μg/ml) for 16 h.

Serum, proteins, and Abs

Normal human serum (NHS) was collected from five healthy individuals, pooled, and stored at −80°C until use. Factor B-depleted human serum (Δfactor B-HS) was bought from Merck Chemicals (Nottingham, U.K.). Pra1 was cloned and expressed in *Pichia pastoris* (23). *S. aureus* Efb was cloned in the expression vector PET200D and expressed as an N-terminal His-tagged protein in *Escherichia coli*. Factor H, factor B, factor D, factor P, factor I, C3, and C3b were obtained from CompTech (Tyler, TX). Gelatin was from Merck (Darmstadt, Germany), human serum albumin (HSA) was bought from Nutritional Biochemicals (Cleveland, OH), and BSA from Sigma-Aldrich (Taufkirchen, Germany). Polyclonal Pra1 antiserum was raised in rabbits with purified rPra1 (23). Goat anti-rabbit, rabbit anti-mouse, and rabbit anti-goat sera labeled with Alexa Fluor-488 (Invitrogen, Karlsruhe, Germany) were used as secondary antisera for flow cytometry and confocal microscopy. C3b/iC3b surface deposition was determined with polyclonal goat C3 antiserum (CompTech). HRP-conjugated rabbit anti-goat, HRP-conjugated rabbit anti-mouse, as well as HRP-conjugated swine anti-rabbit sera were obtained from Dako (Glostrup, Denmark). Polyclonal rabbit anti-C3a serum and polyclonal goat anti-factor H serum were purchased from CompTech.

Generation of factor H-depleted, complement active human serum

A total of 100 μl protein A-Sepharose (GE Healthcare, Freiburg, Germany) was incubated with polyclonal goat anti-factor H serum (200 μl) plus 300 μl Dulbecco's PBS (DPBS) buffer (Lonza) overnight at 4°C on a shaker, as described (51). Unbound anti-Factor H was removed by washing twice with DPBS. Then active NHS (500 μl) was added, and the mixture was incubated for 1 h at 4°C. Next, serum was collected by centrifugation and used directly.

Hemolytic assay

NHS (10%) or factor H-depleted, complement active human serum (Δfactor H-HS; 30%) was pretreated with different amount of Pra1 in DPBS (20 μl) for 20 min at 37°C, then added to rabbit or sheep erythrocytes (1×10^7/sample; BioTrend, Köln, Germany) suspended in 80 μl Mg-EGTA buffer specific for AP activation (20 mM/l HEPES, 144 mM/l NaCl, 7 mM/l $MgCl_2$, 10 mM/l EGTA [pH 7.4]) for 30 min at 37°C. Erythrocyte lysis was determined at 414 nm.

ELISAs

Pra1 and *S. aureus* Efb (0.5 μg/well dissolved in carbonate-bicarbonate buffer) were immobilized onto a microtiter plate (MaxiSorb; Nunc, Wiesbaden, Germany) at 4°C overnight. After washing, nonspecific binding sites were blocked with 0.2% gelatin for 2 h at room temperature (RT). Then C3 and C3b (1 μg/well in 100 μl DPBS) were added and incubated for 1.5 h at RT. Wells were washed with DPBS buffer containing 0.05% Tween 20, and the primary polyclonal anti-C3 serum was added for 1 h at RT, followed by HRP-conjugated secondary rabbit anti-goat serum. Bound proteins were detected using tetramethylbenzidine as a substrate (BioTrend, Köln, Germany). The reaction was stopped with 2 M H_2SO_4, and the OD was determined at 450 nm using an ELISA plate reader (SpektraMax 190; Molecular Devices, Sunnyvale, CA). C5a ELISA was performed by using a C5a ELISA kit (DRG Instruments, Marburg, Germany), according to the manufacturer's instructions.

Analysis of C3a and C5a generation

For activation of AP, NHS (7.5%) or Δfactor H-HS (7.5%) was incubated with heat-treated *C. albicans* (1×10^7) or zymosan (1 mg/ml) in the presence or absence of Pra1 in AP-specific buffer (3 mM $MgCl_2$ and 1 mM EGTA in DPBS) (100 μl). After incubation for 30 min at 37°C, the supernatant was separated by SDS-PAGE and transferred to a membrane, and C3a levels were analyzed by Western blotting using polyclonal rabbit anti-C3a serum or by ELISA (TECOmedical, Bünde, Germany). For C5a generation, NHS was activated by zymosan in presence or absence of Pra1 in AP-specific buffer (3 mM $MgCl_2$ and 1 mM EGTA in DPBS), and then C5a generation was quantified by ELISA.

Analysis of C3 convertase of the AP

The inhibitory effect of Pra1 on C3 convertase formation was analyzed as described, with slight modifications (52). Briefly, NHS (30%) was activated by zymosan (3 mg/ml) for 30 min in HBS^{2+} buffer (20 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$ [pH 7.4]). After washing, the particles were incubated in DPBS for 30 min at 37°C (to dissociate C3 convertases). Then either C3b-coated zymosan or factor B (40 μg/ml), factor D (1 μg/ml), and factor P (4 μg/ml) were preincubated with Pra1 for 15 min at RT and combined together. After 30-min incubation at 37°C, C3bBb levels were analyzed by flow cytometry using Bb mAb (Quidel, San Diego, CA).

The ability of Pra1 to dissociate a preformed C3 convertase was assayed, as described (53). Briefly, C3b (250 ng/well) was coated on microtiter plates; factor B (500 ng), factor D (25 ng), and factor P (1 μg) were added; and the mixture was incubated for 1 h at 37°C. After washing, Pra1 (1.0, 2.0, 5.0, 10.0 μg in 100 μl buffer) or factor H (0.1, 0.2, 0.5, 1.0 μg in 100 μl buffer) was added and incubated for 1 h at RT. Following additional washing steps, C3 convertases attached on the plate were quantified with polyclonal goat anti-factor B serum (Calbiochem, Darmstadt, Germany).

To analyze whether Pra1 binds and stabilizes C3 convertase formed on the zymosan surface, zymosan (1 mg/ml) or *C. albicans* was incubated with NHS (7.5%) in AP-specific buffer. The particles were pellet and washed, and different amounts of Pra1 (1, 2, and 5 μg in 100 μl buffer) were added for 30 min at 37°C. Bound Pra1 was determined by flow cytometry using a Penta-His Ab (1:100) and an Alexa Fluor-488–labeled rabbit anti-mouse (1:200) as a secondary antiserum. In parallel, 10 μl supernatant from each sample was treated under the reducing condition, separated by SDS-PAGE, transferred to a membrane, and then detected by Western blotting using a Penta-His Ab.

To analyze whether Pra1, by binding to C3 in the fluid phase, inhibits C3 cleavage, C3 convertase was preformed on zymosan surface, as described (52). C3, preincubated with Pra, was added to and incubated with this preformed C3 convertase for 30 min at 37°C. The supernatant was separated by SDS-PAGE, and C3a level was analyzed by Western blotting using polyclonal rabbit anti-C3a serum or by ELISA.

Analysis of Pra1 interaction with C3 in solution

To analyze complex formation of Pra1 and C3 in solution, Pra1 used at increasing concentrations (i.e., 0.25, 0.5, 1, and 2 μg) was incubated with constant amounts of C3 (1 μg) in DPBS (100 μl) for 30 min at RT. Then Pra1-C3 complexes were attached to an ELISA plate that was coated with polyclonal C3 antiserum raised in goats (1:5000) for 1 h at RT. After washing, Pra1 complexed to C3 was identified with a polyclonal Pra1 antiserum raised in rabbits (1:2000), followed with swine anti-rabbit serum.

In addition, native gel electrophoresis was used to further prove the Pra1-C3 interaction, and to visualize Pra1-C3 complex formation. Pra1, used at increasing amounts (0.25, 0.5, 1, 2 μg), or HSA (negative control) was incubated with constant amounts of C3 (1 μg) in DPBS (50 μl) for 30 min. Then the samples (20 μl/sample) were separated by 8% native gel at 4°C and bands were visualized by silver staining.

C3b/iC3b deposition on yeast surfaces

Heat-treated *C. albicans* (1×10^7) was incubated with NHS (7.5%), Δfactor H-HS (7.5%), or Δfactor B-HS (7.5%) in the presence or absence of Pra1, or *S. aureus* inhibitor Efb (used as a positive control upon AP activation) (52, 54) for 30 min at 37°C in AP-specific buffer or dextrose–gelatin–veronal buffer (2.5 mM Veronal buffer [pH 7.4], 75 mM NaCl, 2.5% dextrose, 0.05% gelatin, 0.15 mM Ca^{2+}, 0.5 mM Mg^{2+}) (100 μl reaction volume). After centrifugation, the cells were washed with DPBS and kept at RT for 30 min to dissociate C3 convertases. Deposition of C3b/iC3b on *C. albicans* surface was determined by flow cytometry (LSR II; BD Biosciences, Heidelberg, Germany) using a polyclonal goat anti-C3 serum and an Alexa Fluor-488–labeled rabbit anti-goat serum. For confocal microscopy, the C3b/iC3b-opsonized *C. albicans* cells prepared as above were further stained with DAPI (10 μg/ml) for 10 min at RT and fixed, and then C3b opsonization, DAPI, and differential interference contrast were examined by confocal microscopy (LSM 510; Carl Zeiss, Oberkochen, Germany).

Adhesion and phagocytosis assays

Adhesion and phagocytosis of C3b/iC3b-opsonized *C. albicans* were assayed by flow cytometry. Monocytic cells (1×10^6/well) were stimulated with PMA (2 μg/ml) in 24-well culturing plate (Greiner Bio-One, Solingen, Germany) for 16 h. Then the cells, which were kept in FCS-free RPMI 1640 medium, were stained with Vybrant 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO; Invitrogen, Karlsruhe, Germany) (1:100) for 40 min at 37°C. The C3b/iC3b-opsonized *C. albicans* that was prepared as above in presence or absence of different amount of Pra1, or *S. aureus* inhibitor Efb (used as a positive control upon AP activation) (52, 54) was stained with DAPI (10 μg/ml) for 10 min at RT, and then cocultured with DiO-labeled macrophages for 15 min (55). After removal of unattached yeast cells by extensive washing, human cells were detached from the surface of culture plate. Macrophages that adhered and phagocytosed *C. albicans* cells were quantified as double-positive (DiO^+, $DAPI^+$) cells by flow cytometry.

Results

Pra1 binds to C3 and to the activation product C3b

As C3 is the central molecule in the complement system and as several pathogen-encoded complement inhibitors bind to C3 (32, 54), it was of interest to assay whether Pra1 also binds to C3. Pra1 was immobilized, and C3 and C3b were added as ligands. Both C3 and C3b bound to *Candida* Pra1. C3 binding was stronger than C3b (Fig. 1, black columns). In comparison, C3 and C3b also bound to the immobilized *S. aureus* inhibitor Efb (Fig. 1, hatched columns).

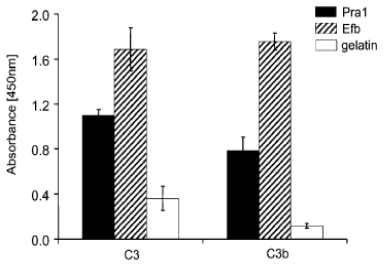

FIGURE 1. Pra1 binds to C3 and to C3b. Binding of C3 and the activation product C3b to rPra1 and *S. aureus* Efb (as a positive control) was assayed by ELISA. Pra1, Efb, and gelatin (negative control) were immobilized and after blocking, C3 and C3b were added in the fluid phase. After incubation, binding was assayed using polyclonal goat C3 antiserum. Data represent the mean values ± SD of three separate experiments.

Pra1 is a complement inhibitor

Showing that Pra1, which is secreted by both yeast and hyphal forms of *C. albicans*, binds to C3 and C3b suggested a complement-regulatory activity of Pra1. In a hemolytic assay with NHS and rabbit erythrocytes, Pra1 inhibited erythrocyte lysis by ~50% upon AP activation (Fig. 2*A*). Pra1 was recently identified as a factor H-binding surface protein (23); we therefore asked whether Pra1-mediated complement inactivation and factor H recruitment are related or two separate effects. To this end, Δfactor H-HS was preincubated with Pra1, then added to sheep erythrocytes suspended in Mg-EGTA buffer. After incubation, erythrocyte lysis was measured. Pra1, used at 20 μg/ml, efficiently inhibited erythrocyte lysis also in Δfactor H-HS by ~90% (Fig. 2*B*, *column 4*). A similar effect of Pra1 on complement inactivation was detected using rabbit erythrocytes and Δfactor H-HS (Supplemental Fig. 1). Thus, Pra1 is an AP complement inhibitor, and the inhibitory effect is independent from factor H binding.

Pra1 inhibits C3a and C5a generation

As Pra1 binds to C3, as well as C3b, we hypothesized that Pra1 affects complement activation at the level of C3 convertase. To prove this, first NHS was activated either by *C. albicans* or by zymosan in presence or absence of Pra1. After incubation, the reaction mixtures were separated by SDS-PAGE, and C3a generation was followed by Western blotting. Pra1 inhibited C3a formation upon AP activation by *C. albicans* (Fig. 3*A*, *upper panel*, *lanes 2–5*) or by zymosan (Fig. 3*A*, *lower panel*, *lanes 2–5*). This effect was dose dependent. At 100 μg/ml, Pra1 completely inhibited C3a release (Fig. 3*A*, *upper and lower panels*, *lanes 5*). Thus, Pra1 inhibits AP activation at the level of C3 convertase.

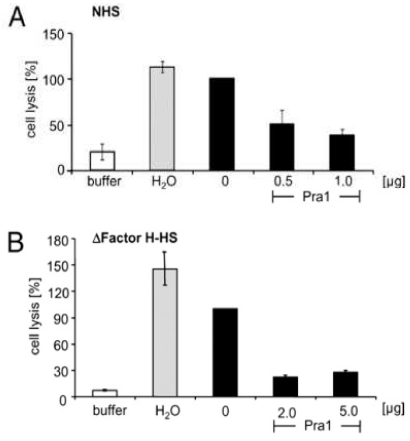

FIGURE 2. *Candida* Pra1 is a complement inhibitor. *A*, Pra1 inhibits rabbit erythrocyte lysis in NHS. NHS (10%) was preincubated with indicated amount of Pra1 (*columns 4* and *5*), and then rabbit erythrocytes suspended in Mg-EGTA buffer were added. After incubation, cell lysis was measured at 414 nm. The background lysis is shown as buffer control (*column 1*); the maximal lysis is shown by H_2O control (*column 2*). *B*, Pra1 inhibits sheep erythrocyte lysis in Δfactor H-HS. ΔFactor H-HS (30%) was preincubated with Pra1 (20 and 50 μg/ml), and then sheep erythrocytes suspended in Mg-EGTA buffer were added. After incubation, cell lysis was measured at 414 nm. Complete erythrocyte lysis was shown by H_2O control (*column 2*). The background lysis was determined by the buffer control (*column 1*). Data represent the mean values ± SD of three separate experiments.

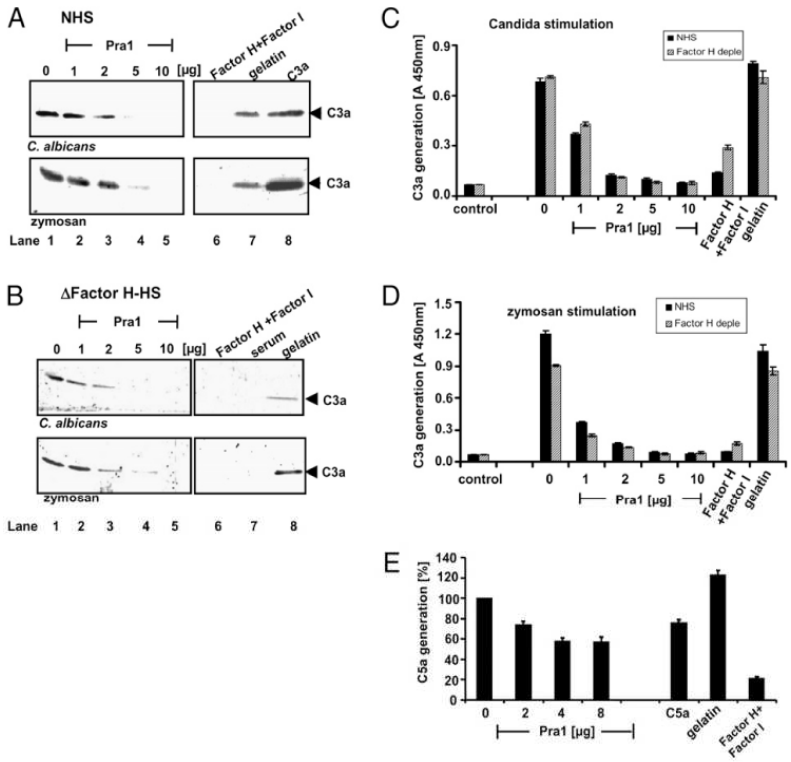

FIGURE 3. Pra1 inhibits C3a and C5a generation. *A*, Pra1 inhibits C3a generation. NHS was activated by *C. albicans* (*upper panel*) or by zymosan (*lower panel*) in the presence of indicated amounts of Pra1 (*lanes 2–5*) using AP-specific buffer. Following incubation, supernatants were separated by SDS-PAGE and transferred to a membrane, and C3a was visualized by Western blotting using polyclonal rabbit anti-C3a serum. Factor H and factor I were used as a positive control (*lanes 6*), and gelatin as a negative control (*lanes 7*). Mobility of purified C3a is shown in *lanes 8*. *B*, Pra1 inhibits C3a generation also in Δfactor H-HS. Again, *C. albicans* (*upper panel*) or zymosan (*lower panel*) was added to Δfactor H-HS in the presence or absence of indicated amounts of Pra1 (*lanes 2–5*). Following reaction, C3a generation was detected by Western blotting. Factor H and factor I were used as the positive control (*lanes 6*), and gelatin as a negative control (*lanes 8*). Serum without activation was separated in *lanes 7*. The data show a representative result of three separate experiments. *C* and *D*, Effect of Pra1 on C3a generation shown by ELISA. NHS (black columns) or Δfactor H-HS (hatched columns) was activated by incubation with *C. albicans* (*C*) or zymosan (*D*) in the presence of different amounts of Pra1, and C3a generation was determined by ELISA. Pra1 dose dependently inhibited C3a generation. Factor H and factor I, which were used as a positive control, efficiently inhibited C3a generation, whereas gelatin, used as a negative control, showed no effect. *E*, Pra1 inhibits C5a generation. NHS was activated by zymosan (1 mg/ml) in presence or absence of indicated amounts of Pra1 (*columns 2–4*). C5a generation in supernatants was quantified by ELISA. Gelatin as a negative control did not inhibit C5a generation (*column 6*). However, factor H and factor I as a positive control inhibited C5a generation (*column 7*). Data represent the mean values ± SD of three separate experiments.

To confirm that this inhibitory effect of Pra1 on C3 conversion is independent of factor H binding, again the inhibitory effect of Pra1 on complement activation was analyzed in Δfactor H-HS. Pra1 still blocked C3a generation when the AP was activated by incubation of either *C. albicans* (Fig. 3*B*, *upper panel*) or zymosan particles (Fig. 3*B*, *lower panel*) in Δfactor H-HS. This effect was dose dependent, and at 50 μg/ml Pra1 almost completely inhibited C3a generation (Fig. 3*B*, *upper and lower panels*, *lanes 4*). Thus, the inhibitory effect of Pra1 on C3a generation is independent of factor H binding, indicating that Pra1-mediated complement inhibition and factor H recruitment are two separate effects.

To further prove the effect of Pra1 on the C3a generation, NHS or Δfactor H-HS was activated by *C. albicans* or zymosan in the presence or absence of Pra1, and then the C3a generation was further analyzed by ELISA. Upon incubation of *C. albicans* in NHS (Fig. 3*C*, black columns) or Δfactor H-HS (Fig. 3*C*, hatched columns), Pra1 inhibited C3a generation in a dose-dependent manner. At 20 μg/ml, Pra1 almost completely inhibited C3a generation (Fig. 3*C*). Similarly, upon incubation of zymosan in NHS (Fig. 3*D*, black columns) or Δfactor H-HS (Fig. 3*D*, hatched columns), Pra1 inhibited C3a generation in a dose-dependent manner.

As *Candida* Pra1 inhibits C3a generation, we asked whether this secreted fungal protein also affects C5a generation. NHS was activated by zymosan, and generation of C5a in the supernatant was quantified by ELISA. Again, upon AP activation, Pra1 blocked C5a release, and this effect was dose dependent. At 40 μg/ml, Pra1 inhibited C5a generation by 43% (Fig. 3*E*, *column 3*). Thus, Pra1 also inhibits C5a generation upon AP activation.

Pra1 does neither affect C3 convertase formation, nor dissociate a preformed C3 convertase

To define how Pra1 blocks AP activation at the level of C3 convertase, we asked whether Pra1 by binding to surface-deposited C3b or other components (factor B, factor D, and factor P) affects C3 convertase formation. To this end, Pra1 was preincubated with C3b-coated zymosan, and then factor B, factor D, and factor P were added. In parallel, Pra1 was incubated with factor B, factor D, and factor P in fluid phase and then added to C3b-coated zymosan. Following 30-min incubation and extensive washing, C3 convertase formation was determined on the surface of zymosan particles by flow cytometry using Bb mAb. In this set up, Pra1 did not affect C3 convertase formation (Fig. 4*A*, black and gray columns).

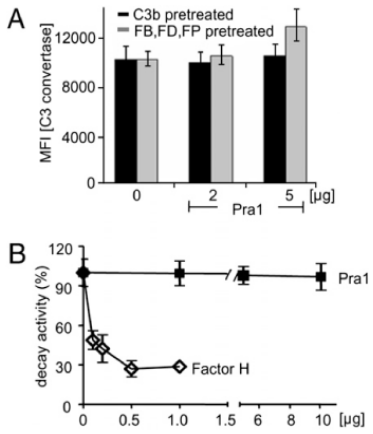

FIGURE 4. Pra1 does neither affect C3 convertase formation, nor dissociate a preformed C3 convertase. *A*, Pra1 does not affect C3 convertase formation. C3b-coated zymosan was pretreated with Pra1, and then combined with FB, FD, and FP (black columns). In parallel, FB, FD, and FP were pretreated with Pra1, and then added to C3b-coated zymosan (gray columns). After incubation, C3 convertase formation on zymosan surface was determined by flow cytometry using Bb mAb. *B*, Pra1 lacks decay-accelerating activity to dissociate a preformed C3 convertase. C3 convertase was assembled on the surface of microtiter plates, and then Pra1 (black squares) or factor H (white diamonds) was added. After incubation, C3 convertase levels were analyzed by ELISA using polyclonal FB antiserum. Data represent the mean values ± SD of three separate experiments. FB, factor B; FD, factor D; FP, factor P; MFI, mean fluorescence intensity.

We next investigated whether Pra1 exerts decay-accelerating activity and dissociates a preformed C3 convertase. To this end, a C3 convertase was assembled on the surface of a microtiter plate using purified complement components. Pra1 was added, and after incubation the remaining C3 convertases were identified using polyclonal goat anti-factor B serum. Pra1 did not dissociate the preformed C3 convertase (Fig. 4*B*, black squares). In contrast, factor H, as a decay-accelerating factor, did dissociate the preformed convertase (Fig. 4*B*, white diamonds). Thus, Pra1 does neither inhibit C3 convertase formation, nor does Pra1 dissociate a preformed C3 convertase.

Pra1 inhibits complement activation in fluid phase and binds C3 in solution

As Pra1 does not inhibit C3 convertase formation, and also lacks decay activity to dissociate a preformed C3 convertase, we asked whether Pra1, as a C3b-binding protein, binds to and stabilizes a surface-attached C3 convertase. To this end, a C3 convertase was assembled on zymosan surfaces with NHS. After washing, Pra1 was added to surface-assembled C3 convertases and incubated for 30 min. Bound Pra1 were determined by flow cytometry. In this case, Pra1 did not bind to C3 convertase (Fig. 5*A*), but remained in fluid phase (Fig. 5*B*). Similarly, Pra1 did not bind to a C3 convertase assembled on the surface of *C. albicans* (data not shown). This suggests that Pra1 inhibits complement activation in fluid phase. To further prove this hypothesis, Pra1 was first incubated with either NHS or Δfactor H-HS, and then rabbit or sheep erythrocytes were added. After incubation, both rabbit and sheep erythrocytes that were protected from lysis by Pra1 were washed and incubated with polyclonal Pra1 antiserum to detect whether Pra1 bound to C3b assembled on the surface of erythrocytes. Again, Pra1 was not detectable on the surface (data not shown). Based on these results, we conclude that Pra1 inhibits complement activation in fluid phase, which is different from the factor H recruitment by surface-expressed Pra1.

Showing that Pra1 inhibits complement activation in fluid phase, we hypothesized that Pra1 binds to C3 substrate in fluid phase and by blocking C3 cleavage inhibits complement activation. To prove this, Pra1 and C3 complex formation in solution was analyzed by ELISA and by native gel electrophoresis. Pra1 and C3 were incubated in solution for 30 min at room temperature, and then the complexes were captured by a polyclonal goat C3 antiserum that was coated to a microtiter plate. After washing, Pra1, complexed to the C3, was identified with a polyclonal Pra1 antiserum. When C3 concentrations were kept constant (1 μg/100 μl) and the concentration of Pra1 was increased, Pra1 showed dose-dependent binding to C3 (Fig. 5*C*).

To further prove Pra1-C3 complex formation, again Pra1 and C3 were incubated in solution, and then the complexes were separated by native gel electrophoresis and visualized by silver staining. The Pra1-C3 complex was identified as a band with a mobility of ~250 kDa. This mobility fits with the predicted molecular masses of Pra1 (58 kDa) and C3 (194 kDa) in nonreduced form. This band was only detectable when both Pra1 and C3 were present (Fig. 5*D*, *lanes 3–6*), but it was not detected when single protein (either C3 or Pra1) or HSA were used (Fig. 5*D*, *lanes 2*, *7*, and *8*). The intensity of this 250-kDa band correlated with the amount of Pra1 (Fig. 5*D*). In this assay, additional bands of slower mobility were detected both for C3 and for Pra1. These bands are considered multimers of either C3 or Pra1. Thus, Pra1 binds C3 and forms a complex in solution.

Pra1, by complexing C3 in fluid phase, inhibits further C3 cleavage by a surface-attached C3 convertase

Knowing that Pra1 complexes C3 in solution, we further tested whether Pra1, by binding to C3 in solution, prevents C3 cleavage by a preformed, surface-attached C3 convertase. C3 was preincubated with soluble Pra1 in fluid phase, and then added to a C3 convertase that was assembled on a zymosan surface. After incubation, C3a generation was determined by Western blotting and by ELISA. In this set up, Pra1 inhibited C3a generation in a dose-dependent manner (Fig. 6*A*, *lanes 2–4*, 6*B*), and used at 20 μg/ml (0.34 μM), Pra1 inhibited C3a generation completely (Fig. 6*A*, *lane 3*, 6*B*, *column 3*). Thus, Pra1, by complexing C3 in solution, blocks C3 cleavage and thus inhibits further complement progression and amplification.

Pra1 inhibits C3b/iC3b deposition on C. albicans *surface*

C3b/iC3b is an important complement effector that is generated upon complement activation. C3b/iC3b deposited onto a surface of a particle or microbe mediates phagocytosis and efficient removal of a tagged particle or microbe (15). As a complement inhibitor, Pra1 blocks complement activation at the level of C3. To define whether and how this complement inhibitory effect of Pra1 favors and correlates with *C. albicans* immune escape, we first asked whether Pra1 limits C3b/iC3b deposition on the surface of yeast cells. To this end, the role of Pra1 on C3b/iC3b surface deposition was assayed following incubation of yeast cells with NHS. Upon AP activation, Pra1 inhibited C3b/iC3b deposition, as demonstrated by confocal microscopy (Fig. 7*A*) and by flow cytometry (Fig. 7*B*). This effect was dose dependent. Pra1 blocked C3b/iC3b surface deposition by ~62% in NHS (Fig. 7*B*) and by 67% in Δfactor H-HS (Fig. 7*C*) at a concentration of 100 μg/ml. *S. aureus* Efb (52, 54), as a positive control at 40 μg/ml (equal molar concentration with 100 μg/ml Pra1), inhibited C3b/iC3b surface deposition by ~61% in NHS (Fig. 7*B*, hatched column) and by 72% in Δfactor H-HS (Fig. 7*C*, hatched column), respectively.

Pra1 inhibits AP activation by blocking C3 conversion. As C3 convertases of both AP and CP/LP are functionally and structurally related, we hypothesized that Pra1 also inhibits CP/LP activation. Yeast cells were incubated in Δfactor B-HS to allow CP/LP activation, and

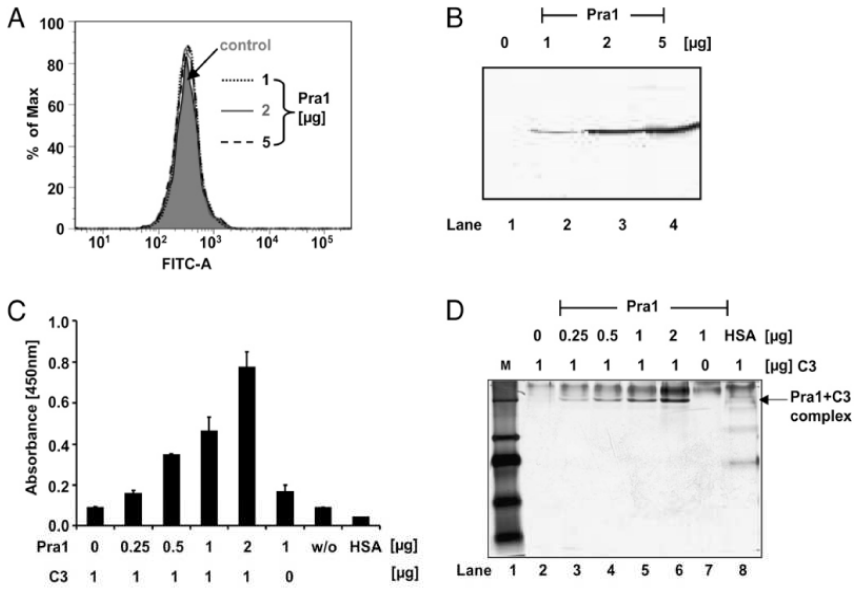

FIGURE 5. Pra1 inhibits complement activation in fluid phase and binds C3 in solution. *A* and *B*, Pra1 does not bind and stabilize a preformed C3 convertase. Zymosan was incubated with NHS; a C3 convertase was formed on zymosan surface. Following addition and incubation of Pra1 with surface-attached C3 convertase, bound Pra1 was assayed by flow cytometry. Pra1 did not bind to C3 convertase on the surface (*A*), but was present in the supernatant (*B*). *C*, Pra1 complexes C3 in solution. Increasing amount of Pra1 was incubated with constant amount of C3 in DPBS for 30 min at RT. Then the sample was added to ELISA plates, which were coated with polyclonal C3 antiserum raised in goats (1:5000) for 1 h at RT. After washing, Pra1, complexed with C3, was identified with a polyclonal Pra1 antiserum raised in rabbits (1:2000), followed with swine anti-rabbit serum. Pra1 forms complexes with C3 in solution, and the complex formation is dose dependent. w/o, represents no ligand incubation, only for the Ab control. HSA was used a negative control. *D*, Pra1-C3 complex formation is further confirmed by native gel electrophoresis. Pra1, used at increasing amounts, was incubated with constant amounts of C3 in DPBS for 30 min. Then the sample was separated by 8% native gel at 4°C, and bands were visualized by silver staining. When both Pra1 and C3 are present, a complex band with a mobility of ~250-kDa was visualized. With increasing amount of Pra1, this band becomes stronger (*lanes 3–6*). In the presence of HSA (*lane 8*), or when either Pra1 or C3 was absent (*lanes 2* and *7*), no band of this mobility was identified. *A*, *B*, and *D* show a representative result of three separate experiments; *C* represents the mean values ± SD of three separate experiments.

the effect of Pra1 on C3b/iC3b surface deposition was followed by flow cytometry. Again, Pra1 inhibited C3b/iC3b deposition on the yeast surface (Fig. 7*D*). Also, this effect was dose dependent, and at 100 µg/ml Pra1 inhibited C3b/iC3b deposition by ~44% (Fig. 7*D*, blue curves). Thus, Pra1, by blocking C3 conversion, inhibits C3b/iC3b surface deposition upon both AP and CP/LP activation. This inhibitory activity is independent of factor H binding.

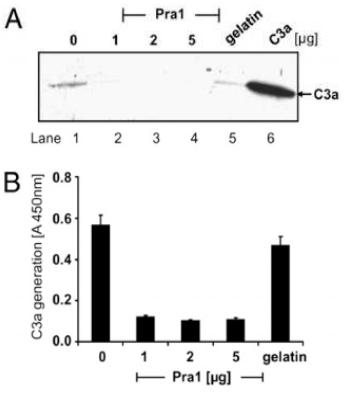

FIGURE 6. Pra1, by complexing soluble C3, blocks further C3 cleavage. *A* and *B*, Pra1 complexes C3 in fluid phase and inhibits C3 cleavage. A C3 convertase was preformed on a zymosan surface, purified C3, preincubated with Pra1 in fluid phase, was added to a preformed C3 convertase for 30 min. The reaction mixture was separated by SDS-PAGE, and C3a level was analyzed by Western blotting using polyclonal rabbit anti-C3a serum (*A*) or by C3a ELISA (*B*). Pra1 inhibited C3a generation in a dose-dependent manner (*A*, *lanes 2–4*, and *B*, *columns 2–4*). Gelatin used as a negative control had no significant effect (*A*, *lane 5*, and *B*, *column 5*). Mobility of purified C3a is shown in *A*, *lane 6*. *A*, A representative result of three separate experiments. *B*, The mean values ± SD of three separate experiments.

Pra1 inhibits C3b/iC3b-mediated adhesion and uptake of C. albicans *by human macrophages*

As C3b/iC3b opsonization mediates the adhesion and phagocytosis of the pathogenic yeast by human macrophages, we hypothesized that Pra1 affected this *Candida*–macrophage interaction by blocking C3b/iC3b generation on yeast surfaces. To prove this, *C. albicans* was incubated in NHS or Δfactor B-HS in the presence or absence of Pra1, or *S. aureus* Efb, used as a positive control upon AP activation (52, 54). After washing, the pellets were stained with DAPI and added to DiO-labeled human macrophages. Human macrophages alone were identified as single-positive cells (DiO^+, $DAPI^-$) (Fig. 8*A*, 8*B*, dotted graphs; *A*, for AP; *B*, for CP/LP; *panels I*, *upper left*, *quadrant 1* [*Q1*]). Following coincubation, macrophages with adhering or phagocytosed yeast cells were identified as double-positive cells (DiO^+, $DAPI^+$) (Fig. 8*A*, 8*B*, *panels II*, *Q2*). Pra1, by inhibiting C3b/iC3b surface deposition, reduced the number of double-positive macrophages. This effect was dose dependent (Fig. 8*A*, 8*B*, *panels III–V*, *Q2*). Upon AP activation, Pra1 inhibited both adhesion and uptake of opsonized yeast cells by human macrophages by 54.0% (Fig. 8*C*) at 100 µg/ml. The *S. aureus* complement inhibitor Efb, used at 40 µg/ml (about equal molar concentration with 100 µg/ml Pra1), inhibited both adhesion and phagocytosis by ~40%. Similarly, upon CP/LP activation, Pra1 inhibits such an interaction by 34.8% at 100 µg/ml (Fig. 8*D*). In summary, the fungal complement inhibitor Pra1, by inhibiting C3b/iC3b surface

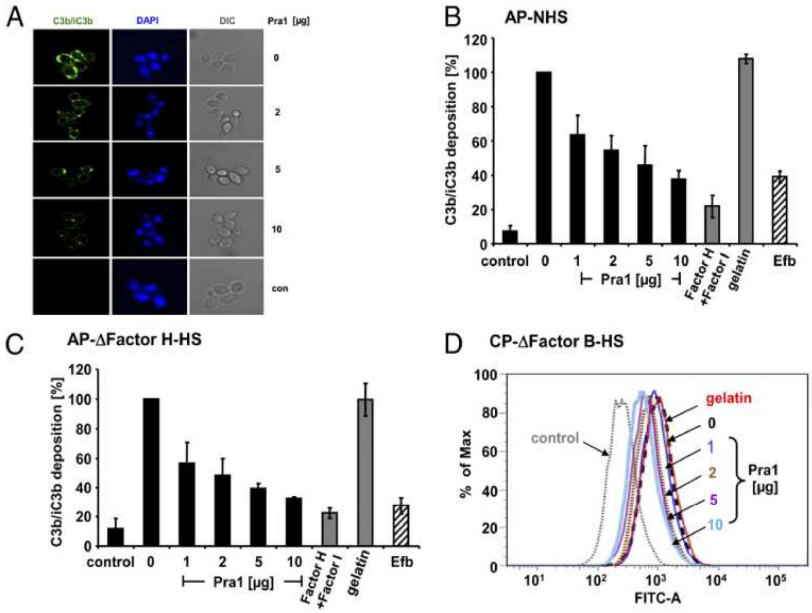

FIGURE 7. Pra1 inhibits C3b/iC3b deposition on *C. albicans* surface. *A*, C3b/iC3b deposition on yeast surface was demonstrated by confocal microscopy upon AP activation in NHS. Heat-treated *C. albicans* cells were incubated with NHS in presence or absence of indicated amounts of Pra1 for 30 min at 37°C in AP-specific buffer. Following incubation with the polyclonal goat anti-C3 serum and an Alexa Fluor-488–labeled rabbit anti-goat serum, cells were stained with DAPI and fixed. C3b/iC3b staining (green), DAPI (blue), and differential interference contrast images, which show the shape and the position of the cells, were recorded by LSM. Cells without incubation with serum were used as a control. *B*, Deposition of C3b/iC3b on the surface of yeast cells was indicated by flow cytometry upon AP activation in NHS. The cells were treated as described above, and surface-bound C3b/iC3b was detected by flow cytometry following incubation with the polyclonal goat anti-C3 serum and an Alexa Fluor-488–labeled rabbit anti-goat serum as a secondary antiserum. Cells without incubation with the serum were used as a control. Factor H, together with factor I, and Efb were used as positive controls. Gelatin was a negative control. *C*, Pra1 also inhibits C3b/iC3b surface deposition in Δfactor H-HS upon AP activation. Again, *Candida* cells were incubated with Δfactor H-HS in presence or absence of Pra1. After washing, C3b/iC3b surface deposition was analyzed by flow cytometry using polyclonal goat anti-C3 serum. *C. albicans* without incubation with serum was used as a negative control. Factor H, together with factor I, and Efb were used as positive controls. Gelatin was a negative control. *D*, Pra1 inhibits C3b/iC3b deposition on the surface of *C. albicans* upon CP/LP activation. *C. albicans* was incubated in Δfactor B-HS in presence or absence of indicated amounts of Pra1. C3b/iC3b deposition on *C. albicans* surface was analyzed by flow cytometry. Gelatin used as a negative control had no effect on C3b/iC3b deposition. *A* and *D* show a representative result of three separate experiments; *B* and *C* represent the mean values ± SD of three separate experiments.

deposition, reduces complement-mediated adhesion and uptake of opsonized *Candida* cells by human macrophages.

Discussion

In this study, we identified Pra1, which is secreted by human pathogenic yeast *C. albicans*, as a potent fungal complement inhibitor. Pra1 binds C3 in solution and blocks the further cleavage of C3 to C3b and C3a by surface-attached C3 convertases of both AP and CP/LP. Thus, Pra1 inhibits the complement amplification loop, further progression, inflammation, as well as adhesion and phagocytosis of the yeast cells. Pra1 is, to our knowledge, the first fungal complement inhibitor identified from human pathogenic yeast *C. albicans*.

rPra1 binds predominantly to C3 (Fig. 1) and does not interact with the C3 convertase (Fig. 5). This interaction is of functional relevance: as C3, complexed with *Candida* Pra1, is neither cleaved by a C3 convertase of the AP nor by that of the CP/LP. This effect results in inhibition of the generation of anaphylatoxins C3a and C5a (Fig. 3, Supplemental Fig. 2), as well as C3b/iC3b surface deposition (Fig. 7). Consequently, *Candida* Pra1, by inhibiting C3a and C5a release, has an anti-inflammatory effect, and by blocking C3b/iC3b surface deposition reduces attachment and uptake of yeast cells by human macrophages (Fig. 8). Thus, the fungal inhibitor Pra1, by controlling important complement reactions, favors survival of the yeast in an immunocompetent host.

Candida Pra1 was initially identified as a fibrinogen-binding protein (46) and was recently characterized as a fungal factor H, FHL-1, and plasminogen-binding surface protein (23). In this work, we identified a new function of Pra1. Pra1 is a potent complement inhibitor that blocks complement activation at the level of C3 (Figs. 3, 6). Factor H, as an AP complement regulator, prevents C3 convertase formation and also dissociates a preformed C3 convertase of the AP (56). It was therefore relevant to demonstrate that Pra1-mediated blockade of complement activation is distinct from factor H recruitment. Pra1 showed the same inhibitory activity on sheep and rabbit erythrocyte lysis (Fig. 2*B*, Supplemental Fig. 1), as well as C3a release and C3b/iC3b surface deposition (Figs. 3, 7*C*) in Δfactor H-HS, compared with NHS. The inhibitory effect of Pra1 on CP/LP activation further proves the specific Pra1 functions as factor H controls the AP, but not the CP/LP. Therefore, Pra1 and factor H-mediated complement inactivation are two separate and independent functions.

Pra1 displayed the complement inhibitory effect in the fluid phase, which correlates with the role of the secreted Pra1 and is separated from surface Pra1-mediated complement evasion by recruitment of factor H onto yeast surface. Pra1, secreted by *C. albicans*, complexes C3 in solution (Fig. 5), inhibits C3 cleavage by a preformed C3 convertase (Fig. 6), and consequently blocks further formation of the complement amplification loop and downstream effector functions. This soluble Pra1-mediated complement

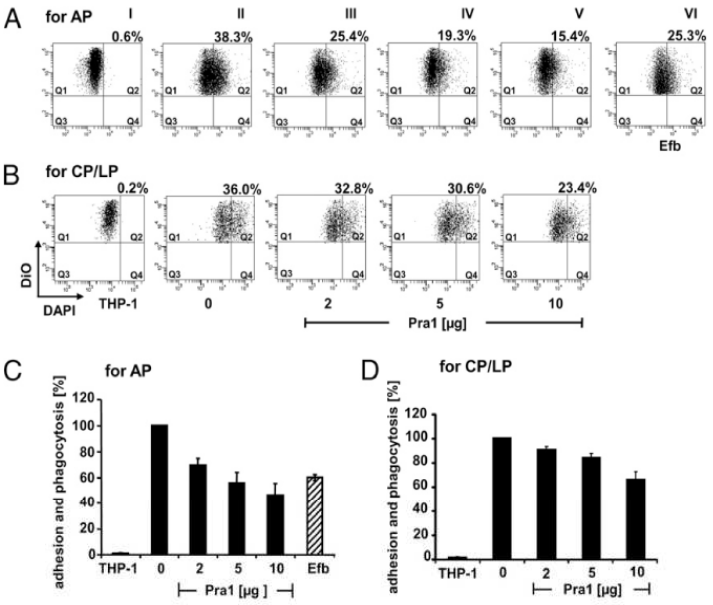

FIGURE 8. Pra1 inhibits C3b/iC3b-mediated adhesion and phagocytosis of yeast cells by human macrophages. C3b/iC3b-opsonized, DAPI-stained yeast cells were incubated with DiO-labeled THP-1 for 15 min. After washing, macrophages alone were detected as single-positive cells (DiO^+, $DAPI^-$) (dotted graphs, *A* for the AP, *B* for CP/LP, *panels I*, *Q1*). THP-1 with adhered and phagocytosed *C. albicans* cells were identified as double-positive cells (DiO^+, $DAPI^+$) (*A*, *B*, *panels II*, *Q2*). Pra1 reduced the number of double-positive human macrophages in a dose-dependent manner (*A*, *B*, *panels III–V*, *Q2*). Efb (used at 4 μg, equal molar with 10 μg Pra1) was used as a positive control (*A*, *panel VI*). Data show a representative result of three separate experiments. *C* and *D*, The mean percentage of Pra1 inhibition on adhesion and phagocytosis from three separate experiments is shown. Pra1, used at 10 μg, reduced both attachment and uptake of opsonized yeast cells by human macrophages by 54% upon AP activation (*C*, *column 5*). *S. aureus* Efb, used at a comparable molar with 10 μg Pra1, inhibited both adhesion and internalization by ~40% (*C*, *column 6*). Similarly, upon CP/LP activation, Pra1 inhibited both adhesion and phagocytosis by ~34.8% at 10 μg (*D*, *column 5*).

inactivation enhances complement control in the direct vicinity of the yeast and generates an additional protective layer for the yeast against host complement attack. The C3 blocking effect of Pra1 is similar to Efb-C (the C3-inhibitory domain of Efb) from the Gram-positive bacterial *S. aureus*, which also binds C3 and C3b and blocks C3 cleavage by the C3 convertase, thereby blocking downstream activation of the complement response (54). However, it is also reported that *S. aureus* Efb binds to C3b containing convertases and blocks complement activation (52). Further investigations will show whether Pra1 and Efb-C, although generated by two different organisms, have overlapping functions. *Candida* Pra1 definitely acts differently from *S. aureus* inhibitor Staphylococcal complement inhibitor, which blocks complement activation by stabilizing C3 convertases (40). As C3 convertases of the CP/LP and the AP are functionally and structurally related, a similar mechanism is also suggested for the inhibition of Pra1 on the CP/LP activation.

Pra1 also inhibits C5a generation (Fig. 3*E*, Supplemental Fig. 2). However, as Pra1 already blocks complement activation at the level of C3, it is currently unclear whether the blockade of C5a release is a direct effect by inhibiting the assembly of a C5 convertase, or a site effect due to the inhibition of C3 conversion and a blockade of the complement amplification loop. Independent of the exact mechanism, *Candida* Pra1-mediated inhibition on C3a and C5a generations presumably prevents inflammatory effector functions, like recruitment of immune effector cells to the sites of infection. Thus, secreted Pra1 forms an anti-inflammatory environment that is beneficial for the pathogen and favors *C. albicans* survival (57, 58).

C3b or iC3b, when deposited onto the microbial surfaces, is recognized by CRs, that is, CR1 and CR3, which are expressed on the surface of human phagocytic cells. This favors both adhesion and phagocytosis (Supplemental Fig. 3). Both effects can be dangerous for pathogens (15). As a potent complement inhibitor, Pra1 inhibits C3b/iC3b surface deposition upon complement activation. The inhibitory effects are of different intensities (by 62% for the AP, by 44% for the CP/LP) (Fig. 7). By decreasing C3b/iC3b surface deposition, Pra1 interferes with C3b/iC3b-mediated adhesion and phagocytosis (Fig. 8) and aids in *C. albicans* immune escape. As multiple other receptors also play a role for recognition of *C. albicans* by macrophages, such as TLRs or dectin-1 (59–61), the inhibition on C3b/iC3b-mediated adhesion and phagocytosis by Pra1 is only partial.

Pra1 is secreted by both *Candida* yeast cells and hyphae, and is identified in culture medium. The semiquantitative approach revealed that within 24 h, 10 million yeast cells generate ~5 μg Pra1 (~50 μg/ml; Supplemental Fig. 4). This concentration correlates with the biological effect observed in this study for rPra1 (10–100 μg/ml). During infection and tissue invasion, local Pra1 levels may even be higher as Pra1 expression is upregulated upon hyphal induction (62). This provides further evidence for the biological relevance of Pra1 in vivo. Thus, locally produced Pra1 represents a potent fungal complement inhibitor that efficiently controls and modulates host complement attack.

Pra1 is both secreted and localized at the surface of both *C. albicans* yeast and hyphae, and does also bind to human cells (23, 46, 47). Such different localizations allow *Candida* Pra1 to act at different sites, as follows: 1) as a secreted protein, Pra1 complexes C3 in solution, inhibits C3 conversion by C3 convertases of both AP and CP/LP, and blocks further complement amplification and progression and downstream immune responses; 2) as a surface protein, Pra1 binds human complement regulators factor H, FHL-1, and plasminogen for complement evasion and tissue invasion (23); and 3) Pra1 also binds to the surface of human cells via CR3 receptors (47). This binding may result in a modulation of the intracellular signaling and affect additional immune effector functions.

Thus, as a multifunctional fungal complement inhibitor, Pra1 apparently forms multiple protective layers that shield the human pathogenic yeast *C. albicans* from the different host complement and immune attacks. Pra1 provides an example for the multiplicity and complexity of the immune escape that are contributed by one single fungal virulence factor. A detailed understanding of these multiple roles of Pra1 allows to define new strategies to interfere with and fight against *Candida* infection.

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Disclosures

The authors have no financial conflicts of interest.

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4.3 The pH-regulated antigen 1 of *Candida albicans* interacts with C4b-binding protein (C4BP) and mediates fungal contact with human endothelial cells.

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The pH-regulated antigen 1 of *Candida albicans* interacts with C4b binding protein (C4BP) and mediates fungal contact with human endothelial cells*

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Running title: *Candida* Pra1 mediates immune evasion and tissue invasion

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Key words: *Candida* Pra1, C4BP, complement evasion, contact

Candida albicans **binds and utilizes human complement inhibitors, such as C4b-binding protein (C4BP), Factor H and FHL-1 for immune evasion. Here, we identify *Candida* Pra1 as the first fungal C4BP binding protein. Recombinant Pra1 binds C4BP and Pra1-C4BP interaction is of ionic nature. The Pra1 binding domains within C4BP were localized to the complement control protein domain 4 (CCP4), CCP7 and CCP8. C4BP bound to Pra1 maintains complement inhibitory activity. C4BP binding was increased about 2 fold in an Pra1 overexpressing *C. albicans* strain and was decreased by 22 % in a Pra1 knock out strain. In addition Pra1 surface expression was up-regulated upon co-cultivation of *C. albicans* with human non-phagocytic endothelial-, epithelial cells and phagocytic monocytes as shown by flow cytometry.**

C. albicans **surface Pra1 mediated fungal adhesion and invasion to non-phagocytic endothelial cells via binding to a new receptor on human cell surface instead of CR3, as shown by flow cytometry. In addition, Pra1 expression and sequence variation was tested in clinical *C. albicans* isolates. Pra1 expression levels of clinical isolates were either higher or comparable to that of the wild type strain SC5314. The sequence of *PRA1* gene was rather conserved. A homozygous nucleotide exchange (A73G) that causes an Asn25Asp exchange on the protein level was identified in all tested strains. In summary, *C. albicans* Pra1, by binding C4BP mediates complement control, and by acting as a bridging protein facilitates contact of *C. albicans* with human endothelial cells and favors *C. albicans* for the tissue invasion.**

INTRODUCTION

Candida albicans is a dimorphic human pathogenic fungus, which frequently causes superficial infections. *C. albicans* can cause systemic, as well as disseminated infections that are most commonly observed in patients who receive immunosuppressive therapy or

long-term catheterization (1). The pathogenicity of *C. albicans* is due to the ability to inactivate human innate immune attack and to overcome host tissue barriers. In this study, we aimed to identify surface proteins that are central for *C. albicans* innate immune escape, as well as invasion of human epithelial or endothelial cells.

The human complement system forms the first defense line of innate immunity. Upon infection microbes are immediately attacked by this highly efficient human immune system (2,3). Complement can be activated via three major pathways. The classical pathway is mainly induced by antibodies bound to target structures or by C- reactive protein and the lectin pathway is activated by binding of mannose-binding lectin and ficolins to mannan-containing structures on surfaces (4-7). The alternative pathway is initiated spontaneously and continuously by randomly generated C3b, and activated C3b can bind directly to any surface and initiate an amplification loop of the alternative pathway (8,9). Complement activation is controlled by several fluid-phase and surface bound inhibitors, such as C4BP, a 570 kDa plasma glycoprotein, which is the major fluid-phase classical/lectin pathway inhibitor (10), and Factor H and FHL-1 which are the major fluid-phase alternative pathway inhibitors (11-15). These inhibitors act as cofactors for serine protease Factor I mediated cleavage of C4b or C3b (16-18). These soluble regulators do also bind to the surfaces of host cells and protect them against complement mediated damage. However, microbial pathogens can also mimic the host surface to acquire complement regulators on their surface for complement evasion (3).

Different human cell types function differently to protect the host against microbial infection and tissue dissemination. Endothelial cells that cover blood vessels form a cellular lining that can control the transit of white blood cells and the passage of materials into and out of the bloodstream. In addition, endothelial cells provide a physical barrier that separates blood from tissue and prevents pathogenic microbes to enter into deeper tissue layers for severe systemic infection (19). Similarly epithelial cells that cover internal as well as external body surfaces, provide a physical barrier, produce anti-microbial peptide that can kill microbes and infected cells, which are considered as the innate immune defense (20). Thus during superficial infections and tissue dissemination, *C. albicans* first has to breakdown and overcome such protective cell layers. In addition, many human immune cells, such as neutrophils, macrophages or dendritic cells that function as professional phagocytic cells are recruited to the site of infection upon microbial invading and initiates a direct and fast response, they phagocytose in particular opsonized microbes and release inflammatory cytokines.

C. albicans has developed multiple strategies to control and avoid immune recognition and to overcome tissue layers. For example, *C. albicans* acquires and utilizes complement inhibitor (C4BP, Factor H and FHL-1) to control complement attack (3,5,21). In addition, to invade deeper tissues, *C. albicans* must overcome non-phagocytic host endothelial and epithelial cell barriers (22). Adhesion and invasion to these protective cells are considered as a critical step for pathogenic microbes to overcome endothelial cell layers and disseminate into deeper tissue layers. During mucosal infection, *C. albicans* invades epithelial cells, either by inducing pseudopod formation to initiate passive uptake or by active penetration mediated by secreted aspartyl proteinases (Saps) and lipases, which digest and damage the surface of host epithelial cells and extra cellular matrix components, thus allow the fungus to enter host cells and to cross tissue layers (22-26). Similarly, during hematogenous infection, *C. albicans* invades endothelial cells

by active penetration or passive uptake (23). The *C. albicans* hyphal surface protein Als3 induce endocytosis into endothelial cells by binding to N-cadherin (27).

The pH-regulated antigen 1 (Pra1) of *C. albicans* is glycosylated protein composed of 299 amino acid. Pra1 is located on the fungal surface and is also released by both yeast and hyphae into the culture supernatant (28-30). As a surface protein, Pra1 binds human plasma proteins Factor H, FHL-1 and plasminogen. As a released protein, Pra1 binds back to the fungal surface (31) and also binds to the integrin CR3 receptor that is expressed on the surface of phagocytic leukocytes (29).

In this study, we identify *Candida* Pra1 as the first fungal C4BP binding protein and localize binding domains within C4BP for Pra1. Pra1 binds to human non-phagocytic CR3 deficient endothelial and epithelial cells via a new receptor and mediates *C. albicans* contact with these cells. In addition, Pra1 is expressed in all thirteen *C. albicans* clinical isolates and sequence analysis showed that all tested isolates had a homozygous nucleotide variation exchange at position 73, which results in a likely relevant amino acid variation at position 25.

EXPERIMENTAL PROCEDURES

C. albicans strains, human cell lines and growth conditions

The *C. albicans* wild type strain SC5314 (32), green fluorescent protein (GFP) labeled *C. albicans* (33) and CA14 wild type (34) and a Pra1 over expression strain (from Prof. Rupp, manuscript in preparation) were cultivated in YPD medium (2 % (w/v) glucose, 2 % (w/v) peptone, 1 % (w/v) yeast extract) at 30 °C. Yeast cells were collected by centrifugation and counted with a hemocytometer (Fein-Optik, Bad Blankenburg). Human umbilical vein endothelial cells (HUVEC), human adult low calcium temperature keratinocytes (HaCaT) were grown in DMEM medium and monocytic U937 cells were grown in RPMI 1640 medium (supplemented with 10 % fetal calf serum (FCS), 1 % utraglutamin and 0.55 ‰ gentamicin sulfate) at 37°C in 5 % CO_2.

Antibodies and proteins

Polyclonal Pra1 anti-serum was raised in rabbits by immunization with purified recombinant Pra1 (31). Alexa Fluor®-647 labeled goat anti-rabbit, Alexa Fluor®-647 labeled rabbit anti-mouse, Alexa Fluor®-488 labeled goat anti-rabbit and Alexa Fluor®-488 labeled rabbit anti-goat (Molecular Probes) were used as the secondary anti-sera for flow cytometry and confocal microscopy. For detection of C4b degradation products, a polyclonal goat anti-C4 (CompTech) was used. Horseradish peroxidase (HRP)-conjugated rabbit anti-goat and HRP-conjugated rabbit anti-mouse as well as HRP -conjugated swine anti-rabbit were obtained from Dako. Monoclonal antibody (MAb) 104 and MAb 67 which recognize CCP1 or CCP4 of C4BP, respectively. Recombinant C4BP and its deletion constructs lacking one CCP each time were generated as described (35). Pra1 was expressed in *Pichia pastoris* strains (31). Native, plasma purified C4BP, Factor H, Factor I and C4b were obtained from CompTech. Gelatin was purchased from MERCK and BSA from Sigma.

Direct binding assays

Pra1 or native, plasma purified C4BP, collagen type I (Calbiochem), collagen type III and type IV (BD Biosciences) (0.5 µg/well in carbonate-bicarbonate buffer) were immobilized onto microtiter plates (MaxiSorb, Nunc) at 4 °C overnight. After washing, nonspecific binding sites were blocked with gelatin (0.2 % in Dulbecco's Phosphate Buffered Saline (DPBS)) for 2 h at room temperature (RT). After washing, full-length recombinant C4BP, various C4BP deletion constructs lacking one CCP domain at a

time (△CCP1, △CCP2, △CCP3, △CCP4, △CCP5, △CCP6, △CCP7, and △CCP8) or Pra1 were added (1 µg/well in 100 µl DPBS) and incubated for 1.5 h at RT. Wells were washed with DPBS-T buffer (DPBS containing 0.05 % tween 20) and specific antibody (anti C4BP or anti Pra1) was added for 1 h at RT. After washing with DPBS-T, HRP-conjugated secondary anti-sera were added and incubated for 1 h at RT. After addition of substrate o-phenylenediamine dihydrochloride (DAKO), the interaction was stopped by 2 M H_2SO_4. Absorbance signals were measured at 492 nm in a microtiter plate reader (SpektraMax 190, Molecular Devices).

Competition assays

Pra1 (0.5 µg/well) was immobilized onto microtiter plates (MaxiSorb, Nunc) overnight at 4 °C. After washing, nonspecific binding sites were blocked with 0.2 % gelatin for 2 h at room temperature (RT). After washing, Factor H (2.5 µg/ml) and C4BP (Factor H:C4BP, 1:0, 1:1, 1:2, 1:5, 1:10, 1:20), or C4BP (10 µg/ml) and heparin (C4BP:heparin, 1:0, 1:1, 1:2, 1:4, 1:8), or C4BP (10 µg/ml) and C4b (C4BP:C4b, 1:0, 1:1, 1:2, 1:4, 1:8) in DPBS with different mass ratios were added, incubated for 1.5 h, and after washing, bound Factor H or C4BP was detected by polyclonal goat anti Factor H antiserum or monoclonal mouse anti C4BP (MAb 67) antibody, followed by HRP-conjugated secondary anti-sera.

Cofactor assay

Cofactor activity of C4BP bound to Pra1 in the solid phase were assayed as described (21). Briefly, different amount of Pra1 was immobilized onto the surface of a microtiter plate (MaxiSorb, Nunc) overnight at 4 °C. After blocking, C4BP dissolved in DPBS (0.4 µg/well) was added. Following extensive washing with DPBS, C4b (0.4 µg/well) together with Factor I (0.8 µg/well) was applied. This mixture was incubated for 30 min at 37 °C, stopped by addition of reducing buffer and samples were separated by SDS-PAGE, transferred to a PDVF membrane (Roth), and C4b degradation products were visualized by polyclonal goat C4 antiserum followed by a secondary HRP-conjugated anti-goat serum.

Flow cytometry

Human cell lines (HUVEC, HaCaT and U937 cells) were passaged two days before the experiment, followed by 24 h in FCS-free medium. To analyze Pra1 binding, human cells ($1x10^6$/sample) were washed by DPBS, and incubated with 10 µg recombinant Pra1 for 30 min at 37 °C. After washing with 1 % BSA in DPBS, polyclonal Pra1 anti-serum (1:200 in 1 % BSA-DPBS) was added and incubated for 30 min on ice, followed by incubation with Alexa Fluor®-647 labeled goat anti-rabbit for 30 min on ice (in dark). After washing, the fluorescence signal was measured by flow cytometry (LSR II, BD). To compare Pra1 surface expression level on different *C. albicans* strains, wild type strains CA14 and SC5314, a Pra1 over expression strain as well as 13 clinical isolates were cultivated in YPD medium overnight at 30 °C. Cells were washed and incubated with polyclonal Pra1 antis-erum (1:200) for 30 min on ice, followed by an Alexa Fluor®-647 labeled goat anti-rabbit as a secondary antiserum for another 30 min on ice. After washing, fluorescence signal was measured by flow cytometry. For C4BP binding to *C. albicans*, wild type (CA14) and a Pra1 overexpressing *C. albicans* strain were incubated with EDTA-NHS (1:3 dilution of normal human serum (NHS) in DPBS, supplemented with 10 mM EDTA) for 1 h at RT, then *C. albicans* cell pellets were washed with 1 % BSA in DPBS and incubated with polyclonal rabbit C4BP anti-serum for 30 min on ice, followed by Alexa Fluor®-488 labeled goat anti-rabbit as a secondary anti-serum for 30 min on ice. After washing,

nucleotides of *C. albicans* were stained by 4´,6 diamidino-2-phenylindole (DAPI, 10 µg/ml) for 15 min at RT. Then the samples were washed, and examined either by flow cytometry or by laser scanning microscopy (LSM 510, Carl Zeiss).

Laser Scanning Microscopy

To analyze Pra1 binding to HUVEC and HaCaT cells, HUVEC and HaCaT cells were grown to confluency in 4 well slide chambers (Lab-Tek®) in DMEM medium, followed by FCS free medium for 24 h. Monolayer cells were incubated with 5 µg Pra1 for 30 min at 37 °C in 5 % CO_2. After washing, the cells were fixed with 3 % paraformaldehyde and blocked overnight (1 % BSA in DPBS), then polyclonal rabbit Pra1 antis-erum (1:200) was added and incubated for 30 min on ice, followed by incubation with Alexa Fluor®-488 labeled goat anti-rabbit (1:200) as a secondary with a combination of wheat germ agglutine (WGA) for the cell membrane staining and DAPI for 30 min on ice. After washing, the samples were examined by LSM.

Co-cultivation of C. albicans and human cells

Wild type *C. albicans* (SC5314) ($1x10^7$/well) were co-cultivated with HUVEC, HaCaT and monocytic U937 cells ($1x10^6$/well) or with culture medium (as negative controls) for 2 h at 37 °C in 5 % CO_2, then Pra1 expression level at *C. albicans* surface was analyzed by flow cytometry using polyclonal goat Pra1 anti-serum, followed by Alexa Fluor®-647 labeled goat anti-rabbit serum.

Adherence and invasion of C. albicans into endothelial cells

Adherence and invasion of *C. albicans* assayed by LSM were performed as described with modification (36). Briefly, HUVEC cell lines were grown to confluency on 12-mm diameter circular glass cover slips in tissue culture medium at 37 °C in 5 % CO_2, then changed to FCS free medium for 24 h, *C. albicans* wild type strain SC5314 ($1x10^6$) were added and co-cultured for 2.5 h. After washing, the cells were fixed with 3 % paraformaldehyde and then blocked overnight with 1 % BSA in DPBS. The non-internalized microorganisms were stained by first incubating the infected monolayers with a 1:100 dilution of a polyclonal rabbit anti-*C. albicans* serum (BioTrend), followed by an Alexa Fluor®-488 labeled goat anti-rabbit (1:200) in DPBS with 1 % BSA. Next, the endothelial cells were stained by DAPI. After three times washing, the cover slips were mounted inverted on a microscope slide with mount fluor, then examined by confocal microscopy. To assay the effect of Pra1 on the adhesion and invasion of *C. albicans* by human endothelial cells, HUVEC cell lines were grown to confluency on 24 well tissue culture plate (Greiner bio-one), followed by FCS free medium for 24 h. After that, HUVEC was stained by Vybrant® DiD (1:100) (Invitrogen) for 40 min at 37 °C. GFP labeled *C. albicans* (1×10^6) was pre-incubated with different amounts of Pra1 for 30 min at 37 °C to acquire additional Pra1 onto the surface or with different dilution of Pra1 specific antiserum (1:1000, 1:500, 1:200 and 1:100) to block surface expressed Pra1 or pre-immune serum (1:200) as a negative control for 30 min on ice, then added to DiD labeled HUVEC cells for 2.5 h in DMEM medium. After complete washing, cells were detached, interaction of *C. albicans* and HUVEC cells were measured by flow cytometry. The invaded HUVEC cells were quantified by the fraction of double positive cells (DiD^+, GFP^+).

RESULTS

Candida Pra1 binds C4BP

Candida Pra1 has collagen like sequences (37), therefore we asked whether Pra1 binds to the human collagen type I, type III or type IV or to the human complement regulator C4BP. Pra1

bound to immobilized C4BP, but neither to any of the three collagens tested as demonstrated by ELISA (Figure 1*A*). Pra1-C4BP interaction was confirmed using a reverse setting. Recombinant C4BP bound to immobilized Pra1 in a dose dependent manner (Figure 1*B*). Thus, *Candida* Pra1 is a C4BP binding protein.

To characterize the nature of the Pra1-C4BP interaction, the effect of NaCl on binding of plasma purified and recombinant C4BP to immobilized Pra1 was assayed. Native, plasma derived C4BP bound to Pra1 at the salt concentrations of 0-100mM (Figure 1*C*, *black bars 1-2*). At physiological NaCl concentration (150 mM) the interaction was reduced by 69.7 %, compared to the one in the absence of NaCl (Figure 1*C*), and at higher salt concentrations binding was even strongly reduced (Figure 1*C*). In the absence of NaCl, recombinant C4BP bound weaker to Pra1 than plasma purified C4BP (Figure 1*C*). Binding was increased at 50 mM NaCl (Figure 1*C*) and again decreased at higher concentrations (Figure 1*C*, *hatched bars 3-6*). Thus Pra1-C4BP interaction is of ionic nature and the binding intensity is influenced by the salt concentration.

Pra1 binds C4BP and also Factor H (31). Therefore we asked if C4BP and Factor H bind simultaneously to *Candida* Pra1 and if these two inhibitors bind to overlapping or to separate sites within the Pra1 protein. To this end, Pra1 was coated on microtiter plates, a constant amount of Factor H together with increasing concentrations of C4BP were added and bound C4BP and Factor H were detected with specific antibodies. Using constant amount of Factor H (2.5 µg/ml), C4BP binding was increased with increasing concentrations of C4BP (Figure 1*D*, *black bars*). Factor H binding remained constant when C4BP levels were less than 5 µg/ml (Figure 1*D, hatched bars*). Factor H binding slightly decreased using a concentration of 7.5 µg/ml C4BP (five times as Factor H amount) and was further reduced by less than 50 % even with a concentration of 50 µg/ml C4BP (20 times as Factor H amount). This mass ratio of C4BP and Factor H is far beyond the physiological one in plasma (C4BP concentration is half as Factor H). Thus, C4BP and Factor H bind simultaneously to *Candida* Pra1. The two human inhibitors bind mainly to distinct sites within the *Candida* Pra1. This weak inhibitory effect of C4BP shown at non-physiological concentrations, may be due to steric effects as C4BP is a rather large protein of 550 kDa.

Localization of Pra1 binding domains in C4BP

To localize the Pra1 binding domains within C4BP, binding of recombinant C4BP deletion mutants to immobilized Pra1 was determined. Recombinant C4BP is composed of 7 identical α chains held together by disulphide bonds at their C termini. Each α-chain contains eight CCP domains. Full length C4BP, as well as C4BP deletion mutants ΔCCP1, ΔCCP2, ΔCCP3, ΔCCP5 and ΔCCP6 bound to Pra1 with similarly intensity and binding of constructs ΔCCP4, ΔCCP7 and ΔCCP8 were reduced (Figure 2*A*). Thus indicating that C4BP contacts Pra1 via three domains, i.e. CCP4, CCP7 and CCP8 (Figure 2*B, domains marked in black*).

Initially when assaying binding of C4BP deletion mutants to intact yeast cells, CCP1 and CCP2 were identified as the major contact domains (21) (Figure 2*B*, *domains marked in gray*). CCPs1-3 bind to heparin and to C4b. To confirm that domains CCPs1-3 are dispensable for C4BP-Pra1 interaction, Pra1 was immobilized, C4BP was added together with heparin or C4b as inhibitors. C4BP binding to Pra1 was not affected by heparin (Figure 2*C*) nor by C4b (Figure 2*C*). Thus, C4BP-Pra1 interaction is independent of domains CCPs1-3.

C4BP bound to Candida Pra1 displays complement inhibitory activity

To analyze if C4BP bound to Pra1 is functional active, C4BP was bound to immobilized Pra1 using different concentrations. After intensive washing, purified C4b and Factor I were added. Following further incubation, supernatants were collected, separated by SDS-PAGE, transferred

to a membrane and C4b cleavage fragments were identified by Western blotting. C4BP together with Factor I and C4b were used as a positive control (Figure 3*A, lane 1*). C4BP bound to Pra1 showed cofactor activity as indicated by the appearance of the 20 kDa α3-fragment of C4b (Figure 3*B*, *lanes 1-3*). No cleavage products were observed when gelatin was immobilized (Figure 3*B*, *lane 4*) or when C4BP or Factor I were absent (Figure 3*C*, *lanes 1 and 2*). Thus, C4BP bound to Pra1 maintains cofactor activity for Factor I mediated cleavage of C4b.

Overexpression of Pra1 at C. albicans surface increases C4BP binding

To verify whether surface expressed Pra1 is relevant for C4BP acquisition to intact yeast cells, binding of C4BP to a Pra1 overexpressing *Candida* strain was studied. This strain, has about two-fold levels of Pra1 at the surface, compared to the wild type strain CA14 (Figure 4*A*). Upon incubation in NHS, C4BP binding to the Pra1 overexpressing strain was about two fold as the wild type strain (MFI, 549 vs 269) (Figure 4*B*). This enhanced binding was confirmed by confocal microscopy. Following incubation with NHS-EDTA, C4BP specific fluorescence was stronger for the Pra1 overexpressing strain than for the wild type strain (Figure 4*C*). Thus surface expressed Pra1 mediates *C. albicans* acquiring human classical pathway inhibitor C4BP from NHS, thereby aids in complement evasion of human pathogenic fungus *C. albicans.*

Pra1 surface expression is up-regulated upon co-cultivation of C. albicans with human cells

During hematogenous infection, *C. albicans* encounters different cell types, such as human non-phagocytic endothelial and epithelial, as well as phagocytic cells (23). To define whether *Candida* Pra1 mediates *C. albicans* contact with human cells, we first analyzed Pra1 expression level upon co-cultivation of *C. albicans* with human cells. Pra1 surface expression was increased three fold upon co-cultivation with HUVEC cells (MFI, 41267vs 13641) (Figure 5*A*) and about two fold upon co-cultivation with epithelial HaCaT cells (MFI 28273 vs 13641) (Figure 5*A)*, compared to the one from co-cultivation of *C. albicans* with medium. Pra1 expression was also increased more than two fold (MFI 36888 vs 16374) (Figure 5*B*) upon co-cultivation of *C. albicans* with monocytic U937 cells. Based on these results, we hypothesized that Pra1 plays a role for the *C. albicans* infection on human cells.

Pra1 binds to several human cell lines

Pra1 binds to the integrin receptor CR3 that is expressed on the surface of human phagocytic cells, e.g. neutrophils and monocytic THP-1 cells (29). As Pra1 surface expression was up-regulated upon *C. albicans* contact with human endothelial, epithelial and monocytic cells, we asked whether Pra1 also binds to these cells. Human endothelial HUVEC and epithelial HaCaT cells lack the integrin CR3 on their surface, which is in contrast to U937 cells, which express CR3 (*Supplementary figure 2*). Binding of *Candida* Pra1 to human HUVEC and HaCaT cells, as well as to U937 cells was assayed by flow cytometry. Pra1 bound with high intensity to HUVEC and HaCaT cells, that lack CR3 (Figure 6*A and B*) and weakly bound to CR3 expressing U937 cells (Figure 6*C*). Furthermore, binding of Pra1 to the CR3 deficient HUVEC and HaCaT cells was confirmed by confocal microscopy. Pra1 bound to both endothelial- and epithelial cells as shown by the green fluorescence (Figure 6*D and* 6*E*). This binding of Pra1 binds to CR3 deficient human endothelial-, as well as epithelial cells suggests the existence of an additional Pra1 binding surface receptor.

Pra1 enhances C. albicans contact with human HUVEC cells

Upon co-culture of *C. albicans* with human endothelial HUVEC cells, *C. albicans* (green) either adhered or invaded to human HUVEC cells (blue) (Figure 7*A, panels III and IV*), which is considered as a critical step for the pathogenic yeast to overcome endothelial cell layers and disseminate into deeper tissue layers (23). As surface Pra1 is up-regulated upon co-cultivation of *C. albicans* with HUVEC cells, and Pra1 also binds to this endothelial cells, we asked if Pra1 mediates *C. albicans* contact with HUVEC cells. To this end, GFP labeled *C. albicans* were added to DiD labeled HUVEC cells. Upon co-cultivation, 16.6 % of the HUVEC cells had adherent or invaded GFP labeled fungal cells as revealed by the double positive cells (DiD^+, GFP^+) (Figure 7*B*, *panel II*, *Quadrant 2*). HUVEC cells without *C. albicans* were visualized as single positive cells (Figure 7*B*, *Quadrant 1*). When recombinant Pra1 was attached to the surface of GFP labeled *C. albicans* prior to co-cultivation with HUVEC cells (31), the fraction of double positive human HUVEC cells (DiD^+, GFP^+) was increased by 21.7 % (0.25 µg Pra1) and by 55.4% (0.5 µg Pra1), respectively (Figure 7*B, panel III and IV, Q2*). This indicates that Pra1 influences and mediates *C. albicans* contact with human endothelial cells.

To further confirm the role of Pra1 for adhesion and invasion of *C. albicans* into endothelial cells, Pra1 at the yeast surface was blocked using specific Pra1 anti-serum. Upon co-cultivation with the control *C. albicans* (without Pra1 antiserum treatment), 33.2 % of the HUVEC cells had adherent and invaded *C. albicans* as revealed by double positive cells (set as 100 %) (Figure 7*C*). When the GFP labeled *C. albicans* were treated with Pra1 anti-serum prior to co-cultivation with DiD labeled HUVEC cells, the fraction of double positive (DiD^+, GFP^+) cells was decreased (Figure 7*C*). This effect was dose dependent. At a dilution of 1:100, Pra1 specific anti-serum inhibited *C. albicans* adhesion and invasion to HUVEC cells by ca. 51 % (Figure 7*C*). Pre-immune serum showed no effect (Figure 7*C*). Thus, surface expressed Pra1 mediates both adhesion and invasion of *C. albicans* into HUVEC cells that lack CR3 on their surface, which indirectly indicates that the new second Pra1 receptor expressed on HUVEC cell surface is relevant *C. albicans* adhesion and invasion.

Surface Pra1 expression of and sequence variation in clinical C. albicans isolates

Given the multifunctional roles of *Candida* Pra1 and polymorphism of several microbial virulence factors, we characterized Pra1 surface expression, as well as Pra1 sequence variation in clinical *C. albicans* isolates collected from different infected patients. Five clinical strains expressed higher Pra1 levels as compared to *C. albicans wild type* strain SC5314 (MFI 20844), six strains showed comparable surface levels and two other strains had lower level of Pra1 at their surface (Figure 8).

In addition, the sequence of the *PRA1* gene was determined for each clinical isolate. Chromosomal DNA was isolated, the *PRA1* gene was amplified by PCR and sequenced. The nucleotide sequences of *PRA1* for all tested clinical isolates were relatively conserved and similar to the wild type strain SC5314. A total of 16 nucleotide exchanges were identified in 900 nucleotides of the *PRA1* open reading frame (Figure 9*A*). Compared to the referenced sequence of strain SC5314, each clinical isolate had at least two nucleotides exchanged. These changes occurred either in homozygous or heterozygous settings and were identified with different frequencies. The nucleotide exchange A73G was identified in all 13 clinical isolates in homozygosity (Figure 9*A*).

Seven of total 16 nucleotide exchanges result in amino acid changes (Figure 9*B, marked*

in red). Four of the seven amino acid variations (Asp90Glu, Glu101Asp, Ser154Thr, as well as Asp225Glu) represent conservative changes and seem of no or minor significance. However three exchanges in the N-terminus of Pra1, i.e. residues 25, 105 and 111 resulted in both a polar, uncharged Asn- and a non-polar Gly residue to negatively charged Asp. In addition, at position 111 a non polar Ile residue is changed to a polar, uncharged Ser residue (Figure 9*B*, *marked in blue*). The allelic frequency of these three exchanges varies significantly (Figure 9*A*) in 13 isolates. The N-terminal Asn25Asp exchange is detected in all tested clinical isolates with homozygosity (Figure 9*A, solid circle*). The Gly105Asp exchange was identified in one isolate as homozygous- and in three isolates as heterozygous modification and the Ile111Ser exchange was identified in one single isolate as a heterozygous exchange (Figure 9*A*, *dashed circles*).

DISCISSION

In the current study, we identify *Candida* Pra1 as the first fungal C4BP binding protein. In addition, we define the existence of a second Pra1 receptor that is expressed on the surface of human endothelial and epithelial cells that lack Pra1 ligand CR3. Pra1 functions as a contact or bridging protein that mediates *C. albicans* adhesion and invasion into human endothelial cells, thus favors fungal infection. In addition, Pra1 expression and also sequence variation were determined in 13 clinical *C. albicans* isolates, which were derived from different infected patients. All clinical strains show one major sequence variation as compared to referenced strain SC5314. The identification of novel functions makes the multifunctional fungal virulence *Candida* Pra1 an attractive candidate for immune intervention.

C4BP acquisition is a general immune evasion strategy and is used by many pathogens. Pra1 is the first fungal C4BP binding protein identified from a human pathogenic yeast. Both serum derived and recombinant C4BP bind to *Candida* Pra1 (Figure 1*A* and 1*B*), which is of ionic nature and is affected by NaCl (Figure 1*C*). This type of interaction is similar to the Factor H-Pra1 interaction(31), but different from that of the C4BP-streptococcal M proteins interaction, which is of hydrophobic nature (38). C4BP binds *Candida* Pra1 via domains CCP4, CCP7 and CCP8, but not via the N-terminal domains CCPs1-3, which are also used for the heparin and C4b binding. In addition, the complement inhibitory activity of C4BP is localized within CCPs1-3 (18,39), so binding of Pra1 to C4BP via C-terminal domains CCP4, 7 and 8 orientates the C4BP in such a way that the protein better exposes its functional regions. Rather similar attachment of other microbial proteins to C4BP has been defined for ubiquitous surface protein A 1 (UspA 1) and UspA 2 of *M. catarrhalis,* that bind C4BP via domains CCP2 and CCP7 (40), *Haemophilus influenzae* (41) as well as outer membrane protein A (OmpA) of *Escherichia coli* K1 that binds C4BP via domains CCP3 and CCP8 (42). However, streptococcal M protein (38) and *Neisseria gonorrhoeae* porins (43) bind to C4BP via the N-terminal CCP1. Filamentous hemagglutinin of *Bordetella pertussis* (44) bind to C4BP via CCP1 and CPP2. Similarly when analyzing intact *C. albicans*, the relevant binding domains within C4BP were localized to CCP1 and CCP2 for both yeast and hyphal forms (21). This difference between C4BP binding to Pra1 and to intact *Candida* cells can be explained by the existence of an additional C4BP ligand(s) expressed on the surface of *C. albicans*.

The Pra1 overexpressing *C. albicans* strain binds C4BP more efficiently (about 2 fold) than the wild type strain SC5314 (Figure 4). Furthermore, a Pra1 knock out mutant shows reduced C4BP binding (ca. 22 %), but not completely lacked C4BP binding

(Supplementary figure 3). These binding properties indicate that native surface expressed Pra1 mediates *C. albicans* acquisition of C4BP and also suggest the existence of additional C4BP binding proteins at *C. albicans* surface. *C. albicans* can utilize surface Pra1 to acquire human complement inhibitor C4BP. Bound C4BP maintains complement inhibitory activity and acts as a cofactor for Factor I mediated cleavage of C4b (18). This C4b inactivation will inhibit formation of the classical pathway C3 convertase (C4bC2a) on the fungal surface and consequently blocks further progression of the complement cascade, generation of inflammatory products, as well as C3b opsonization. This explains how Pra1 aids *C. albicans* to control and inhibit complement attack.

In addition to complement attack, *C. albicans* surely faces various host cell barriers, such as immune effector cells (i.e. phagocytic neutrophils, macrophages and dendritic cells), as well as non phagocytotic cells (i.e. endothelial and epithelial cells) during the course of superficial and hematogenous infection. Pra1 expression is up-regulated on the fungal surface upon co-cultivation of the yeast cells with human endothelial as well as epithelial cells and also with human monocytes (Figure 5). This is consistent with previously study showing the induction of Pra1 upon *C. albicans* contact with human epithelial cells on a transcription level (45). This enhancement suggests a role of Pra1 for pathogen-host interaction. Pra1 binds to the integrin CR3 (29) and consequently binds to CR3 expressing phagocytic human cells such as U937 cells (Figure *6C*). However, Pra1 also binds to non-phagocytic cells, that lack CR3 (Figure 6 and Supplementary figure 2). Thus demonstrating the existence of a second Pra1 receptor.

As different cell types have distinct roles to protect the host against infection, the ways by which Pra1 mediates fungal evasion and infection via binding to three different cell lines is likely distinct. Here, we show that surface attached Pra1 enhances both adhesion and invasion *C. albicans* to human endothelial cells that lack CR3 (Figure 7*B*). This interaction is blocked by specific Pra1 anti-serum (Figure 7*C*). Thus, surface expressed Pra1 mediates *C. albicans* contact and invade host endothelial cell barriers. Such cellular invasion of *C. albicans* has been demonstrated for porcine vascular explants (46), and also for brain microvascular endothelial cells (1,23)·(47,48). Similar to Pra1, two other surface proteins of *C. albians*, Als3 and Hwp1 also induce fungal adhesion and invasion to host cells (27,49). In addition, this infection feature seems conserved for many microbial pathogens, such as *Streptococcus pneumoniae, Toxoplasma gondii, Citrobacter freundii, Listeria monocytogenes* and *Staphylococcus aureus* also control and stimulate their own invasion by human endothelial cells via surface expressed virulence factors (50-54). To avoid the human phagocyte effect, *C. albicans* secretes Pra1 to fluid phase, secreted Pra1 may act as a decoy, bind to cell surfaces via CR3, thereby inhibit further CR3 mediated phagocytosis and killing of yeast cells. In support of this hypothesis, experimental results show that addition of soluble Pra1 to a mixture of *C. albicans* and neutrophils increases fungal survival (29).

Pra1 surface expression and sequence variation were assayed in clinical *C. albicans* isolates that were collected from different infected patients. Pra1 is expressed at the surface of all tested clinical isolates with variable levels. Five isolates showed higher Pra1 surface levels as compared to the wild type SC5314 strain. According to sequence determination. *PRA1* was identified as a relatively conserved gene and is not as polymorphic as other Factor H/C4BP binding proteins. e.g. such as CRASPs protein of

Borrelia species (55), or M protein from *S. pyogenes* (56). In the nucleotide level, 16 positions among the 897-base pair long coding region are changed in the tested clinical isolates. These nucleotide exchanges occurred either in homozygous or in heterozygous scenarios were identified with different frequencies. Most of the changes do not affect the protein sequence or only cause the conservative amino acids changes. Interestingly, all tested clinical isolates show the same homozygous A73G exchange that affects the N-terminus of protein sequence, which likely represents the most relevant residue of Pra1 for *C. albicans* infection.

At present the domain structure of *Candida* Pra1 is not well known. Only a collagen-like motive is suggested in Pra1 sequence, which may be involved in the anchoring, attaching and colonization of *C. albicans* onto the extra-cellular matrices (37). So far the binding sites for the various human complement inhibitors have not been mapped in this fungal protein. It is therefore of interest to define whether the N-terminal position at 25 includes a C4BP binding region and if this Asn-Asp exchange affects the interaction of Pra1 with this and also other ligands, therefore favors *C. albicans* infection.

Candida Pra1 is an effective and multi-functional fungal virulence factor. Different functions of Pra1 correlates with its distinct localizations. ***As a yeast surface protein***, Pra1 by binding to several human complement inhibitors such as C4BP, Factor H, FHL-1, mediates immune and complement evasion (31); ***As a secreted protein***, Pra1 complexes C3 in solution, inhibits complement activation, and also enhances Factor H mediated complement control (31). ***Bound to human phagocytic cells via CR3***, Pra1 may act as a decoy to block CR3 mediated physiological functions. ***Bound to human non-phagocytic cells via a new undefined receptor instead of CR3***, surface expressed Pra1 mediates *C. albicans* adhesion and invasion into these cells.

The multifunction that are now described for *Candida* Pra1 provides an example for the multiplicity and complexity of the immune escape that are contributed by one single fungal virulence factor. A detailed understanding of these multiple roles of Pra1 allows to define new strategies to interfere with and fight against *Candida* infection.

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FOOTNOTES

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Abbreviations: CCP, complement control protein; C4BP, C4b-binding protein; Pra1, pH-regulated antigen 1; MAb, monoclonal antibody; HRP, horseradish peroxidase; GFP, green fluorescent protein; HUVEC, human umbilical vein endothelial cells; HaCaT, human adult low calcium temperature keratinocytes; DPBS, Dulbecco's Phosphate Buffered Saline; NHS, normal human serum.

FIGURE LEGENDS

Figure 1: *Candida* Pra1 binds C4BP. (A) Pra1 binds to C4BP. C4BP, Collagen type I, III and IV or BSA and buffer as negative controls were immobilized, bound Pra1 was detected with a specific rabbit Pra1 anti-serum, followed by secondary HRP-conjugated anti rabbit serum. (B) C4BP binds to immobilized Pra1 in a dose dependent manner. Pra1 was immobilized, increasing amounts of recombinant C4BP were added in the fluid phase and bound C4BP was detected by MAb 67, followed by secondary HRP-conjugated anti-mouse serum. (C) Pra1-C4BP interaction is of ionic nature. Pra1 was immobilized and binding of either plasma purified- (*black bars*) or recombinant C4BP (*hatched bars*) was assayed in the presence or absence of different concentrations of NaCl in DPBS. Bound C4BP was detected with MAb 67. (D) Competition of C4BP and Factor H for Pra1 binding. Pra1 was immobilized, a constant amount of Factor H (2.5 µg/ml) together with increasing concentrations of C4BP were added in DPBS using different mass ratios (1:0, 1:1, 1:2, 1:5, 1:10, 1:20). Bound Factor H (*hatched bars*) and bound C4BP (*black bars*) were detected either with goat anti Factor H-serum or with mouse anti-C4BP (MAb 67). Data shown represent the means ± standard deviation (SD) of three separate experiments.

Figure 2: Localization of the binding domains within C4BP. (A) Localization of Pra1 binding domains within the C4BP protein. Pra1 was immobilized, and binding of recombinant C4BP and C4BP deletion mutants each lacking one single CCP domain was detected with the monoclonal anti-C4BP antibody (MAb for △CCP1, △CCP2, △CCP3, △CCP5, △CCP6, △CCP7, and △CCP8 or MAb104 for △CCP4). (B) Effect of heparin and C4bp on C4BP binding to Pra1. Pra1 was immobilized, C4BP together with increasing concentrations of heparin (*black bars*) or C4b (*hatched bars*) was added and bound C4BP to Pra1 was detected using MAb 67. Data shown represent the means ± SD of three separate experiments.

Figure 3: C4BP bound to Pra1 displays cofactor activity. C4BP was bound to Pra1 (immobilized with 0.5 µg (*lane 1*), 1 µg, (*lane 2*) and 2 µg (*lane 3*). After intensive washing, purified Factor I and C4b were added. After incubation the reaction mixture was separated by SDS-PAGE, transferred to a membrane and cleavage of C4b was assayed by Western blotting with polyclonal C4 anti-serum. C4BP incubated with Factor I and C4b were used as positive control to identify C4b cleavage fragments (*A*). Cofactor activity of C4BP bound to Pra1 is indicated by the appearance of the 20 kD α3-fragment (B, *lanes 1-3*). No cleavage occurred in the absence of C4BP or Factor I (C, *lanes 2 and 3*), or C4b alone (C, *lane 1*). A representative result is shown out of three independent experiments.

Figure 4: Pra1 overexpression at *C. albicans* surface increases C4BP binding. (A) Pra1 expression levels on different *C. albicans* strains. *C. albicans* wild type CA14 (*dotted black curve*) and a Pra1 overexpressing strain (*black curve*) was incubated with polyclonal Pra1 anti-serum or pre-immune serum (*grey curve*) which was used as a negative control, the fluorescence signal was determined by flow cytometry. The figure shows one representative experiment out of three separate assays. (B) C4BP derived from NHS binds stronger to the Pra1 overexpressing strain as compared to the wild type strain. *C. albicans* was incubated in NHS-EDTA and after washing, bound C4BP binding was detected by flow cytometry using polyclonal rabbit C4BP anti-serum, followed by Alexa Fluor®-488 labeled goat anti-rabbit serum. The data shown represent the mean values ± SD of

three separate experiments. (C) Binding of C4BP to both Pra1 overexpression and wild type strains was confirmed by confocal microscopy. Again, a Pra1 overexpressing (*upper panel*) and wild type (*lower panel*) strains were incubated with NHS-EDTA. After washing, the cells were incubated with polyclonal rabbit anti-C4BP serum, followed by Alexa Fluor®-488 labeled goat anti-rabbit serum. Nuclei were stained with DAPI (10 µg/ml) and examined by confocal microscopy. The figures shows one representative experiment out of three performed.

Figure 5: Up-regulation of Pra1 surface expression upon co-cultivating *C. albicans* with human cells. (A) *C. albicans* was co-cultivated with non phagocytic CR3 deficient HUVEC (*black bar1*) or HaCaT cells (*grey bar 2)* or DMEM medium (*white columns*) for 2 h at 37 °C. Then Pra1 surface expression was analyzed by flow cytometry using polyclonal rabbit Pra1 anti-serum, followed by Alexa Fluor®-647 labeled goat anti-rabbit serum. The data shown represent the mean value ± SD of three separate experiments. (B) Following co-cultivation of *C. albicans* with human CR3 expressing monocytic U937 cells (*black curve*) or RPMI1640 medium (*dotted black curve*). Pra1 surface expression was determined by flow cytometry using specific Pra1 anti-serum, followed by Alexa Fluor®-647 labeled goat anti-rabbit serum. The figure shows one representative experiment out of three performed.

Figure 6: Pra1 binds to human cells. Recombinant Pra1 or buffer (as negative controls) was incubated with CR3 deficient endothelial HUVEC (A), HaCaT (B) and CR3 expressing U937 cells (C). After washing, bound Pra1 was detected by flow cytometry using polyclonal Pra1 anti-serum, followed by Alexa Fluor®-647 labeled goat anti-rabbit serum. In addition, binding of Pra1 to CR3 deficient HUVEC and HaCaT cells was further confirmed by confocal microscopy. HUVEC (D) or HaCaT cells (E) were grown to confluency in four well slides chamber, and then incubated with Pra1. Bound Pra1 was detected with specific Pra1 anti-serum, followed by Alexa Fluor®-488 labeled goat anti-rabbit serum (*green*). Nuclei of the human cells were stained with DAPI (*blue*) and the cellular membranes with wheat germ agglutinin (*red*). After washing, binding was examined by confocal microscopy. The figures show one representative experiment out of three independent experiments performed.

Figure 7: Pra1 enhances invasion of *C. albicans* into HUVEC cells. (A) Interaction of *C. albicans* with HUVEC cells. HUVEC cells grown on glass covers slides were co-incubated with *C. albicans*. After washing, extra-cellular *C. albicans* were stained by a specific cell wall anti-serum together with Alexa Fluor®-488 labeled goat anti-rabbit serum (green). HUVEC cells were stained with DAPI (blue). After washing, cells were examined by confocal microscopy. (B) Pra1 enhances *C. albicans* adhesion and invasion to HUVEC cells. GFP labeled *C. albicans* were pre-incubated with recombinant Pra1, then added to DiD labeled HUVEC cells. After washing, infected HUVEC cells were identified as double positive cells (Q2) as analyzed by flow cytometry. The results show one representative experiment of three performed. (C) Pra1 anti-serum inhibits *C. albicans* adhesion and invasion into HUVEC cells. GFP labeled *C. albicans* was pre-incubated Pra1 anti-serum, used at different dilutions or pre-immune serum and then added to DiD labeled HUVEC cells. After washing, the HUVEC cells with adhered and internalized *C. albicans* were analyzed by flow cytometry (shown the percentage of double positive cells, without serum treating was set as 100 %). Data shown represent the means ± SD of three separate experiments.

Figure 8: Pra1 surface expression on the clinical *C. albicans* isolates. Pra1 surface expression was analyzed on thirteen different clinical *C. albicans* isolates collected from patients at different sites. The individual clinical isolates as well as wild type SC5314 strain were incubated with Pra1 specific antiserum and analyzed by flow cytometry. The figure shows one representative results of the independent experiments.

Figure 9: Sequence variations of *PRA1* in clinical *C. albicans* isolates. *PRA1* gene in each clinical isolate was isolated, amplified by PCR and sequenced to identify gene polymorphisms. As compared to the referenced sequence of SC5314 strain, *PRA1* sequence in clinical isolates was rather conserved. In complete 897-base pair long protein coding regions, 16 residues were exchanged either at homozygous (*A, isolates 1-13, marked in black*) or at heterozygous scenario (*A, isolates 1-13, marked in grey*) with different frequency, of which seven changes also affect the amino acid sequences (*B, marked in red*). Among these amino acids variations, three however resulted in submissions of the neutral uncharged residue Asn_{26-} or of the non polar Gly_{105} residue to negatively charged Asp residues. In addition, non polar Ile_{111} is exchanged by a polar, uncharged Ser residue (*B*, *marked in blue*).

Figure 1

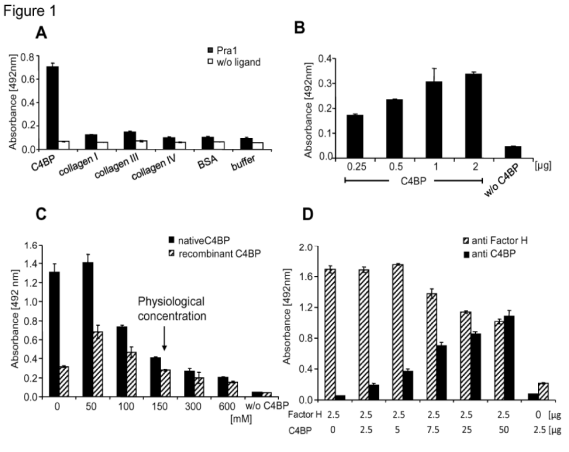


Figure 2

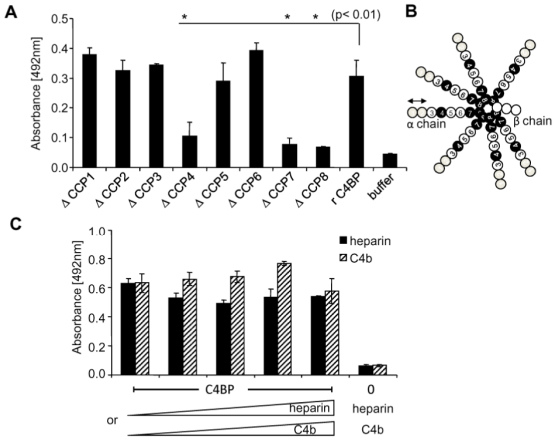


Figure 3

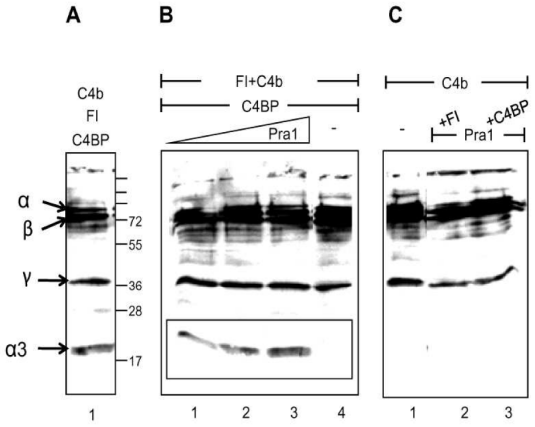


Figure 5

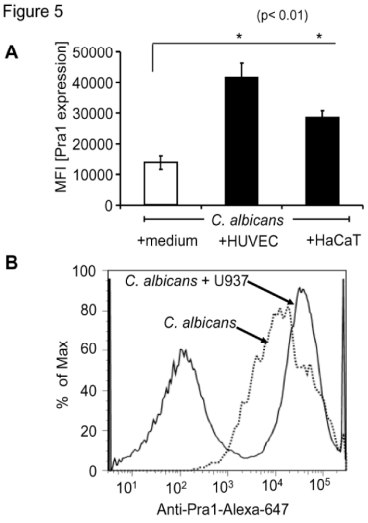


Figure 4

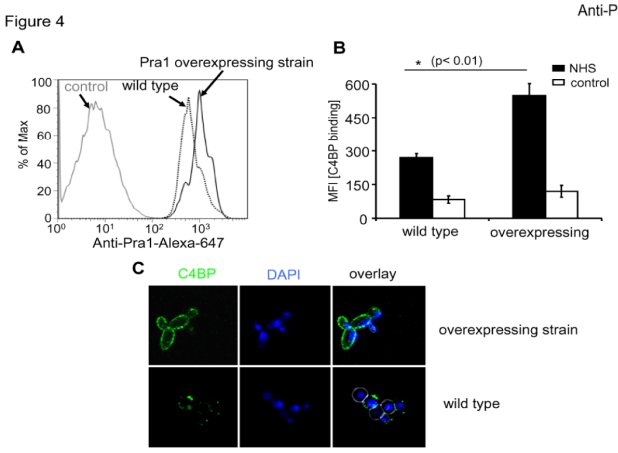


Figure 6

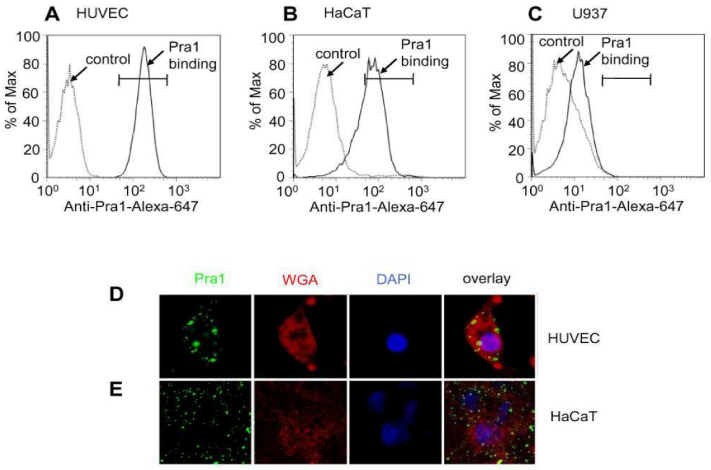


Figure 7

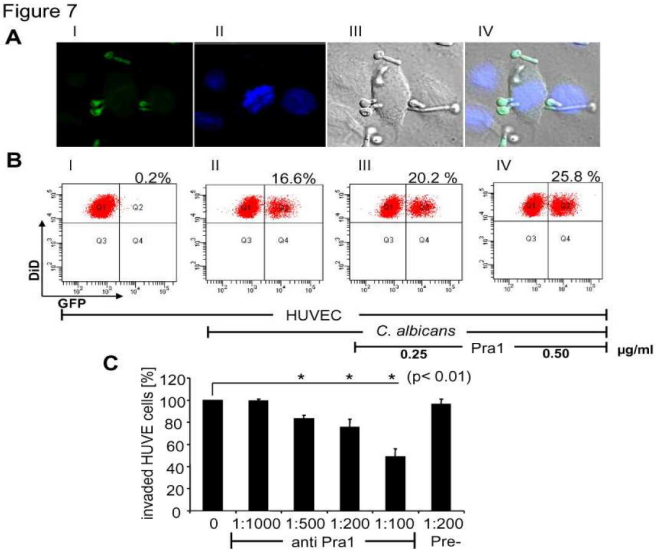
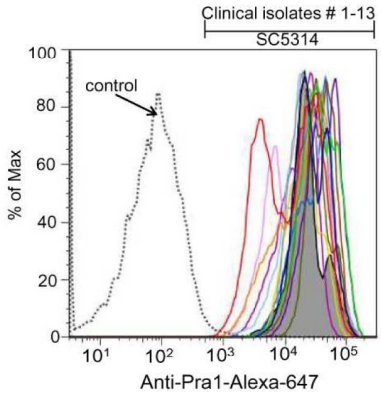


Figure 8



Sample name	MFI[Pra1 expression]
Control	72
SC5314	20844
Clinical # 1	31564
Clinical # 2	33854
Clinical # 3	17197
Clinical # 4	21693
Clinical # 5	18597
Clinical # 6	20577
Clinical # 7	41282
Clinical # 8	22341
Clinical # 9	23043
Clinical # 10	32778
Clinical # 11	12393
Clinical # 12	34493
Clinical # 13	25119

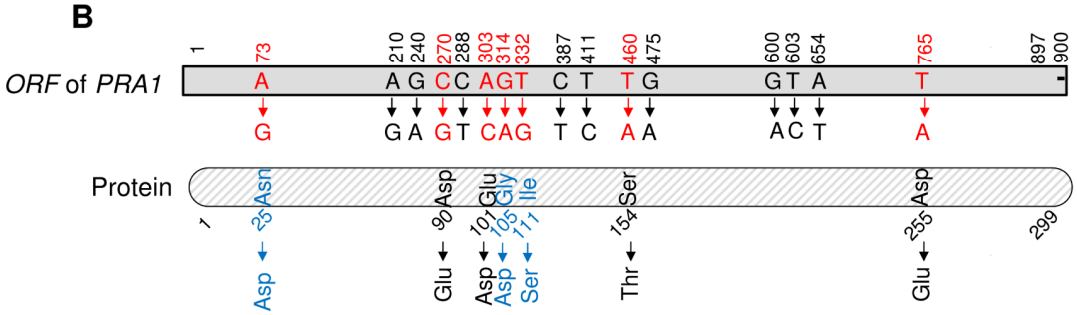
Figure 9

A

Isolate	Infection sites	A73	A210	G240	C270	C288	A303	G314	T332	C387	T411	T460	G475	G600	T603	A654	T765
		Positions in *Candida albicans PRA1*															
1	Oral cavity	GG	GG	GG	GG	CC	AA	AA	TT	TT	TT	TT	GG	AA	TT	TT	TT
2	Oral cavity	GG	AG	GA	CC	CC	AA	GA	TG	CT	TT	TT	GG	GG	TT	AA	TT
3	Oral cavity	GG	GG	GG	GG	TT	CC	GG	TT	TT	CC	AA	AA	AA	CC	AA	TT
4	Oral cavity	GG	AG	GG	CG	TT	AC	GG	TT	TT	CC	TT	GG	GG	TT	AA	TT
5	Oral cavity	GG	AG	GG	CG	CT	AA	GG	TT	CT	TC	TT	GG	GG	TT	AA	TT
6	Tongue	GG	AA	GG	CC	CC	AA	GG	TT	CT	TT	TT	GG	GG	TT	AA	TT
7	Palate	GG	AA	GG	CC	CC	AA	GG	TT	CT	TC	TT	GG	GG	TT	AA	TT
8	Tracheal secretion	GG	AG	GG	CC	CC	AA	GA	TT	CT	TT	TT	GG	GG	TT	AA	TT
9	Inguinal	GG	AG	GG	CC	CT	AA	GG	TT	CT	CC	TT	GG	GG	TT	AA	TT
10	Inguinal	GG	AA	GG	CC	CC	AA	GG	TT	TT	CC	AA	AA	AA	CC	AA	TT
11	Sacral	GG	AG	GG	CC	CC	AA	GA	TT	CT	TT	TT	GG	GG	TT	AA	AA
12	Foot	GG	AG	GG	CC	CC	AA	GG	TT	CC	TC	TT	GG	GG	TT	AA	TT
13	Foot	GG	AA	GG	CC	CC	AA	GG	TT	CT	TT	TT	GG	GG	TT	AA	TT

Homozygous exchange
Heterozygous exchange
No exchange

B



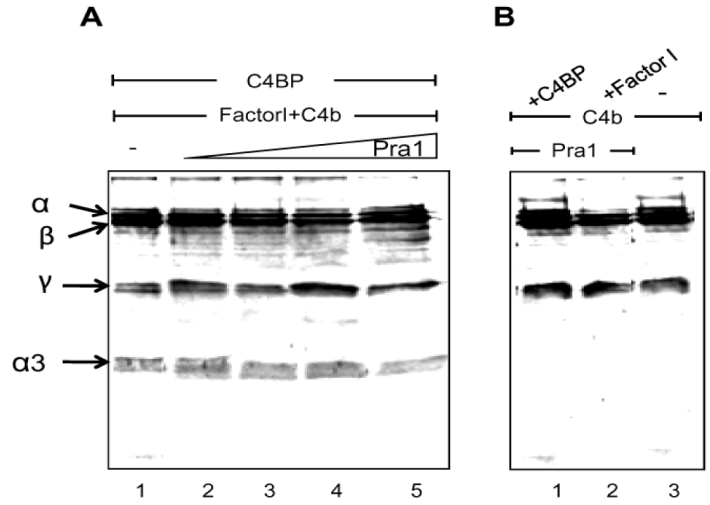
Supplementary information

Suplementary figure 1. Fluid phase cofactor activity was assayed by adding recombinant Pra1 to a mixture containing C4BP (10 µg/ml), Factor I (1 µg/ml), C4b (10 µg/ml). After incubation for 30 min at 37 °C, supernatant were treated by reducing buffer, separated by SDS-PAGE, transferred to a membrane, and C4b degradation products were visualized by polyclonal goat C4 anti-serum and followed with a secondary HRP-conjugated rabbit anti-goat serum. The cofactor activity of the bound C4BP is indicated by the appearance of the 20 kD α3-fragment (*A*, *lanes 1-5*). No cleavage products were observed in the absence of Factor I or C4BP (*B*, *lanes 1 and 2*), or C4b alone (*B*, *lane 3*). The result show one representative experiment of three performed.

Suplementary figure 2. CR3 expression on human cells surface. HUVEC, HaCaT and U937cells were incubated with anti CD11b (*black curve*), anti CD18 (*dotted black curve*), isotype IgG (*grey curve*) (BD, Bioscience) or buffer (*dotted gray curve*) for 30 min on ice (1:50 in 1% BSA in DPBS), followed with Alexa Fluor®-647 rabbit anti-mouse serum (1:100 in 1% BSA-DPBS). HUVEC (A) and HaCaT (B) cells did not express CR3, but U937 (C) did on the surface. The results show one representative experiment of three performed.

Suplementary figure 3. Wild type and Pra1 knock out strain were cultivated in YPD medium. After washing, cell pellets were incubated with EDTA-NHS (30 % NHS in DPBS, supplemented with 10 mM EDTA) for 1 h at RT, after washing, binding of C4BP, Factor H and plasminogen to both strains were analyzed by flow cytometry using polyclonal rabbit C4BP-, polyclonal goat Factor H- or polyclonal goat plasminogen anti-sera, followed by Alexa Fluor®-647 rabbit anti-goat or goat anti-rabbit sera. Binding of C4BP, Factor H and plasminogen to Pra1 knocked out strain (*black bars*) was reduced by ca. 22 %, 9 % and 30 % respectively, compared to the wild type strain (*hatched bars*). The result shows one representative experiment of three performed.

Supplementary figure 1



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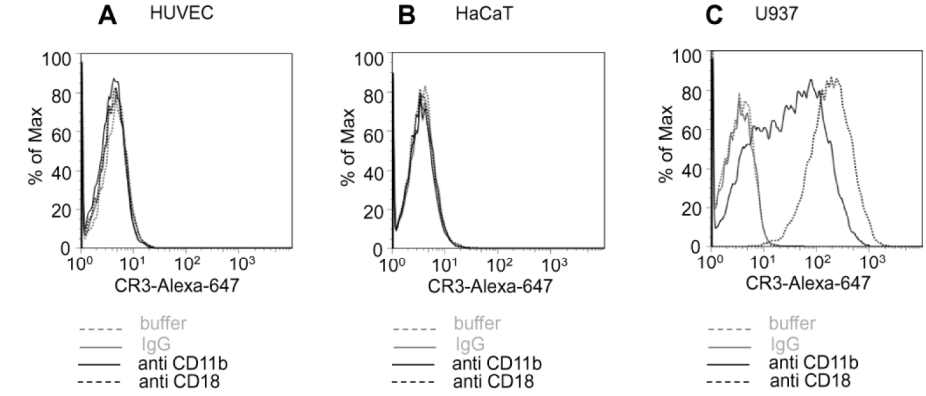
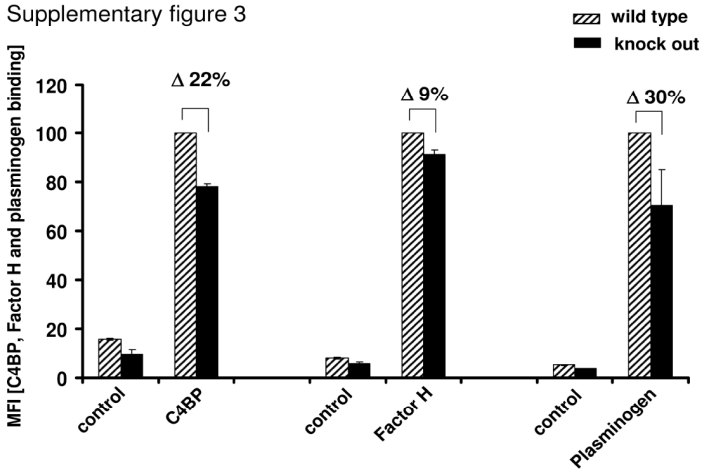


Supplementary figure 3



4.4 Complement regulator Factor H mediates a two-step uptake of *Streptococcus pneumoniae* by human cells.

Vaibhav Agarwal, Tauseef M. Asmat, **Shanshan Luo,** Inga Jensch, Peter F. Zipfel, and Sven Hammerschmidt. *J Biol Chem*. 2010 Jul 23; 285(30):23486-95.

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Complement Regulator Factor H Mediates a Two-step Uptake of *Streptococcus pneumoniae* by Human Cells*

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***Streptococcus pneumoniae*, a human pathogen, recruits complement regulator factor H to its bacterial cell surface. The bacterial PspC protein binds Factor H via short consensus repeats (SCR) 8–11 and SCR19–20. In this study, we define how bacterially bound Factor H promotes pneumococcal adherence to and uptake by epithelial cells or human polymorphonuclear leukocytes (PMNs) via a two-step process. First, pneumococcal adherence to epithelial cells was significantly reduced by heparin and dermatan sulfate. However, none of the glycosaminoglycans affected binding of Factor H to pneumococci. Adherence of pneumococci to human epithelial cells was inhibited by monoclonal antibodies recognizing SCR19–20 of Factor H suggesting that the C-terminal glycosaminoglycan-binding region of Factor H mediates the contact between pneumococci and human cells. Blocking of the integrin CR3 receptor, *i.e.* CD11b and CD18, of PMNs or CR3-expressing epithelial cells reduced significantly the interaction of pneumococci with both cell types. Similarly, an additional CR3 ligand, Pra1, derived from *Candida albicans*, blocked the interaction of pneumococci with PMNs. Strikingly, Pra1 inhibited also pneumococcal uptake by lung epithelial cells but not adherence. In addition, invasion of Factor H-coated pneumococci required the dynamics of host-cell actin microfilaments and was affected by inhibitors of protein-tyrosine kinases and phosphatidylinositol 3-kinase. In conclusion, pneumococcal entry into host cells via Factor H is based on a two-step mechanism. The first and initial contact of Factor H-coated pneumococci is mediated by glycosaminoglycans expressed on the surface of human cells, and the second step, pneumococcal uptake, is integrin-mediated and depends on host signaling molecules such as phosphatidylinositol 3-kinase.**

Streptococcus pneumoniae (pneumococci) colonize as harmless commensals the mucosal epithelium of the human upper respiratory tract. However, pneumococci are also harmful pathogens causing severe infections in humans that are associated with high mortality rates and death. In addition to their ability to cause severe local infections such as otitis media and sinusitis, pneumococci cause life-threatening invasive diseases, including community-acquired pneumonia, sepsis, and meningitis (1–4). Pneumococci have evolved several strategies to adhere to host cells and to evade the host complement and immune attack, both representing prerequisites for pneumococci to disseminate into the lungs and bloodstream or to survive in various host niches. The key bacterial players are virulence determinants that are, with the exception of the toxin pneumolysin, displayed on the pneumococcal cell wall (3, 5, 6). To avoid complement-mediated bacterial lysis, pneumococci recruit, similar to other pathogens, the central complement regulators Factor H and C4b-binding protein (7, 8). The major Factor H-binding protein of *Streptococcus pneumoniae* is the choline-binding protein PspC (pneumococcal surface protein C), which represents a polymorphic surface protein and is termed Hic (factor H-binding inhibitor of complement) in another subset of strains (9–15). The C-terminal choline-binding domain of PspC anchors the protein noncovalently to the phosphorylcholine of the cell wall, whereas the PspC-like Hic (PspC11.4) is covalently anchored to the peptidoglycan of pneumococci after transpeptidase cleavage of the LPXTG motif (16). Both PspC and Hic share in their N-terminal regions a binding domain for the human complement inhibitor Factor H (12, 17, 18). The classical PspC was initially identified as an adhesin mediating adherence to host cells via hexameric motifs located in repeated domains of PspC. These repeats, referred to as R domains, are only present in the classical PspC proteins and interact in a human-specific manner with the ectodomain of the polymeric immunoglobulin receptor (pIgR)[3] of mucosal epithelial cells (9, 19–21). As PspC interacts via two different epitopes with Factor H and the ectodomain of pIgR, this adhesin is able to execute both functions in parallel when displayed on the pneumococcal cell surface (10, 17).

Factor H, which consists of 20 domains referred to as short consensus repeats (SCR), is a single chain plasma glycoprotein and an important fluid-phase regulator of the alternative com-

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[3] The abbreviations used are: pIgR, polymeric immunoglobulin receptor; PI3K, phosphatidylinositol 3-kinase; SCR, short consensus repeat; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DS, dermatan sulfate; CHO, Chinese hamster ovary.

plement pathway (22). Factor H regulates the alternative pathway of complement activation in the fluid phase as well as on host cellular surfaces. Factor H binds to and inactivates C3b in fluid phase; however, Factor H can also bind to eukaryotic cells (23). Factor H surface binding is mediated by the host cellular surface, and molecules such as sialic acids, sulfated polysaccharides (heparins), or glycosaminoglycans. Factor H simultaneously binds both polyanionic surface molecules and surface-bound C3b molecule (24–26).

Factor H has three different C3b- and polyanion-binding sites, which are located in SCR1–4, SCR12–14, and SCR19–20 for C3b and SCR7, SCR13, and SCR19–20 for heparin, respectively (27–31). Factor H also binds dermatan sulfate (DS), which is a sulfated glycosaminoglycan and constituent of various proteoglycans that are present on eukaryotic cell surfaces and in the extracellular matrix (32–35). Moreover, Factor H and Factor H-like protein 1 (FHL-1), which is encoded by an alternatively spliced transcript and consists of the first seven SCR, bind via an RGD sequence in SCR4 to host cells (36). The integrin CR3, also referred to as CD11b/CD18 or α_M/β_2, of human polymorphonuclear leukocytes (PMNs) was identified as the cellular receptor recognizing Factor H (37).

We have previously demonstrated that Factor H binds to the pneumococcal PspC via two regions that are localized in SCR8–11 and SCR19–20 of Factor H (17). In addition, we indicated that pneumococcal-bound Factor H promotes bacterial adhesion and facilitates uptake thereby increasing the numbers of intracellular pneumococci. Although the function of Factor H as a bridging molecule is intriguing, the host cellular receptor(s) and induced signal cascades have not yet been addressed. In this study, we explored the molecular mechanisms that facilitate ingestion of pneumococci by epithelial cells via the Factor H mechanism. We suggest a two-step mechanism for Factor H-mediated pneumococcal invasion into host cells. First, Factor H binds to pneumococci, and bound Factor H is oriented in a way that it can interact with polyanionic molecules on the surface of host cells. This facilitates pneumococcal adhesion, and second, pneumococci exploit a Factor H-integrin complex for invasion into epithelial cells. This invasion process via Factor H requires the dynamics of the actin cytoskeleton and kinase activities of signal transduction molecules.

EXPERIMENTAL PROCEDURES

Cultivation of Pneumococci—S. pneumoniae (NCTC10319, serotype 35A, PspC3.3) were cultured on blood agar plates (Oxoid, Wesel, Germany) at 37 °C and 5% CO_2 or in Todd-Hewitt broth (Roth, Karlsruhe, Germany) supplemented with 0.5% yeast extract (THY) to a density of 5×10^8 colony-forming units ml^{-1} (A_{600} of ~0.5). Binding of Factor H to pneumococci is a general phenomenon, and previous data showed that NCTC10319 can be used to demonstrate the effects (17). NCTC10319 is a low encapsulated, pneumolysin-positive strain and a model strain for *in vitro* cell culture infection studies (17, 38, 39). Cytolytic effects due to pneumolysin are avoided in infections up to 3 h as described earlier (40).

Cell Lines and Culture Conditions—Cultivation of host cell lines was performed as described previously (17). Briefly, human A549 cells (lung alveolar epithelial cells, type II pneumocytes; ATCC CCL-185) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, penicillin G (100 units ml^{-1}), and streptomycin (0.1 mg ml^{-1}) (all from PAA, Germany) at 37 °C and 5% CO_2. A549 cells synthesize heparan sulfate, dermatan sulfate, or chondroitin sulfates but not the polymeric Ig receptor (19, 41). CHO-K1 wild-type cells (ATCC CCL-61, a hamster fibroblast cell line) and stably transfected CHO-CD11b/CD18 (CHO-K1 were stably transfected with cDNA for full-length human integrin complement receptor CR3 (CD11b/CD18)) (42) were cultivated in Ham's F-12 medium (Invitrogen) supplemented with 10% FBS and 2 mM glutamine (PAA). The medium for CHO-CR3 medium was further supplemented with 1 mg ml^{-1} of the antibiotic G418.

Factor H, Antibodies, and Other Reagents—Human Factor H and polyclonal anti-factor H antibodies were purchased from Calbiochem; mouse anti human CD11b antibodies and mouse anti-human CD18 antibodies were purchased from Invitrogen, and monoclonal mouse IgG1 and IgG2 isotype control antibodies were purchased from Ancell (Loerrach, Germany). Purification of rabbit polyclonal anti-pneumococcal IgG (19) was performed by protein A-Sepharose 4B affinity chromatography. Monoclonal antibodies (mAbs) M14, CO2, and C18 employed in blocking experiments were previously mapped to the middle region (M14), SCR19 (CO2), and SCR19–20 (C18), respectively (43, 44). Dermatan sulfate and FITC-heparin were purchased from ICN and Invitrogen, respectively, and heparin and heparinase III were purchased from Sigma. *Candida albicans* pH-regulated antigen 1 (Pra1) was expressed as described previously (45, 46). Cytochalasin D was purchased from MP Biomedicals; nocodazole was obtained from Sigma, and wortmannin, genistein, NSC23766, as well as Y276322 were obtained from Calbiochem. Secramine A, a specific inhibitor of Cdc42, was a kind gift of Tomas Kirchhausen, Immune Disease Institute, Harvard Medical School, Boston, and used as described previously (47–49). *Clostridium difficile* toxins TcdB10463 were kindly provided by Klaus Aktorius and Gudula Schmidt, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Freiburg, Germany (50). The amounts of inhibitors used in this study are not toxic to pneumococci and A549 cells as reported recently (38, 47).

Pneumococcal Host Cell Adherence and Invasion Assay—Pneumococcal host cell adherence and invasion assays were performed as described previously (17). Briefly, A549 cells were seeded at a density of 2.5×10^4 in plain medium either on 24-well tissue culture plates (Greiner, Germany) or on glass coverslips (diameter, 12 mm) when assayed by immunofluorescence and cultivated for 48 h. Confluent monolayers were washed thoroughly and infected for 3 h with pneumococci in 500 μl of Dulbecco's minimal essential medium/HEPES (DMEM/HEPES; PAA) supplemented with 1% FBS at 37 °C using a multiplicity of infection of 50 bacteria per cell. The role of human Factor H for adherence was analyzed by incubating (1×10^7) pneumococci for 20 min with 2 μg of Factor H in a total volume of 100 μl of DMEM/HEPES with 1% FBS at 37 °C prior to host cell infections, and the infection assays were carried out in a total volume of 500 μl after adding the preincubated bacteria, without washing off the unbound Factor H.

Post-infection cells were washed three times with PBS to remove unbound bacteria. The total number of adherent and invasive bacteria was monitored after detachment and lysis of cells with saponin (1% w/v) and plating the bacteria on blood agar. The number of viable intracellular bacteria was quantified by employing the antibiotic protection assay (40). Briefly, epithelial cells were infected with pneumococci (multiplicity of infection of 50), and thereafter, the infected and washed host cells were incubated for 1 h with DMEM/HEPES containing 100 μg ml^{-1} gentamicin and 100 units ml^{-1} penicillin G at 37 °C and 5% CO_2 to kill extracellular and nonadherent pneumococci. Invasive and viable pneumococci were recovered from the intracellular compartments of the host cells by a saponin-mediated host cell lysis (1.0% w/v), and the total number of invasive pneumococci was monitored after plating sample aliquots on blood agar plates, followed by colony formation and enumeration. In inhibition experiments, infection assays were carried out in the presence of soluble heparin, dermatan sulfate, and antibodies or after pretreatment of host cells with 10 milliunits ml^{-1} heparinase III or with various pharmacological inhibitors as described recently (38, 47). The cells were pretreated for 3 h with heparinase III, which cleaves heparan sulfate but does not cleave unfractionated heparin or low molecular weight heparins. Thereafter, the cells were thoroughly washed prior to bacterial infections as described recently (39). Each experiment was repeated at least three times, and results were expressed as mean ± S.D.

Fluorescence Microscopy—Pneumococci attached to host epithelial cells were stained using a polyclonal pneumococcal antibodies (IgG) in combination with a secondary goat anti-rabbit IgG coupled with Alexa Fluor 488 (green) or Alexa Fluor 568 (red) (MoBiTec) (47). Post-infection nonspecific binding sites were blocked with 10% FBS, and before incubating the cells with pneumococcal antibodies (1:100), the infected cell layer was thoroughly washed with PBS. Bound antibodies were detected with an Alexa Fluor-labeled goat anti-rabbit Ig conjugate (MoBiTec, Göttingen, Germany). The glass coverslips were embedded "upside down" in Mowiol, sealed with nail polish, and stored at 4 °C. A confocal laser scanning microscope (Leica TCS SP5 AOBS) and the appropriate CLSM software (LAS AF SP5) were used for the image acquisition.

Flow Cytometric Analysis of Factor H Binding to Pneumococci—Binding of Factor H to viable pneumococci was measured using flow cytometry. Bacteria (1×10^7), cultivated in THY, were incubated in 100 μl of PBS with Factor H in the absence or presence of heparin, which was used as competitor. The suspensions were incubated at 37 °C, and after 30 min the bacteria were washed three times. Factor H bound to pneumococci was then detected by incubating the washed bacteria for 30 min at 37 °C with anti-Factor H antibodies followed by a FITC-conjugated anti-goat Ig antibody (MoBiTec) resulting in fluorescent bacteria. Finally, bacteria were washed, and the fluorescence intensity was analyzed by flow cytometry using a FACSCanto (BD Biosciences). FITC-labeled heparin was used to examine binding of heparin to pneumococci or to Factor H-coated pneumococci. Bacteria were detected using log-forward and log-side scatter dot plot, and a gating region was set to exclude debris and larger aggregates of bacteria. 10,000 bacteria (events) were analyzed for fluorescence using log scale amplifications. The mean fluorescence intensity multiplied by the percentage of PMNs in complex with fluorescent-labeled bacteria was recorded as a measure for binding activity.

Flow Cytometric Analysis of Pneumococcal Association with PMNs—Human PMNs were isolated from peripheral blood of healthy volunteers (Department of Transfusion Medicine, University of Greifswald; oral informed consent was obtained) as described previously (39). Briefly, 1×10^5 PMNs were incubated for 30 min in 100 μl of PBS, 1% FBS with 1×10^6 pneumococci pretreated with or without Factor H. Following bacterial incubations, PMNs were washed once with ice-cold PBS, 0.5% FBS by centrifugation at 280 × *g* for 5 min. The PMNs were then resuspended in 1:100 dilution of rabbit polyclonal anti-pneumococcal IgG and then kept on ice for 30 min. The suspension was washed once with ice-cold PBS, 0.5% FBS and incubated with Alexa Fluor 488-conjugated anti-rabbit Ig antibody (Invitrogen) for 30 min on ice. Finally, PMNs were fixed with 200 μl of PBS, 0.5% FBS, 1% paraformaldehyde. Binding of bacteria (PMN-pneumococci associates) was assessed by flow cytometry using a FACSCanto flow cytometer (BD Biosciences). The geometric mean fluorescence intensity multiplied with the percentage of gated labeled cells was recorded as a measure for pneumococcal binding and uptake.

Statistical Analysis—The binding experiments and infection experiments were performed at least three times, each in duplicate, and the data were expressed as mean ± S.D. Differences in adherence or invasion were analyzed by the two-tailed unpaired Student's *t* test. In all analyses, a p value of <0.05 was considered statistically significant.

RESULTS

Epithelial Cell Surface Glycosaminoglycans Promote Factor H-mediated Adherence of Pneumococci—As Factor H binds heparin and DS, we investigated the role of the glycosaminoglycans heparin and DS on Factor H-mediated adherence of pneumococci to human lung epithelial cells A549. Competitive inhibition experiments were performed in the presence of soluble heparin (50 units/ml), DS (100 μg/ml), or after pretreatment of epithelial cells with heparinase III. Heparin and also DS inhibited Factor H-mediated pneumococcal adherence to A549 cells significantly, although the basal level of adherence in the absence of Factor H was not altered by these two inhibitors (Fig. 1*A*). In contrast, treatment of the lung epithelial cells with heparinase III, which specifically cleaves heparin sulfate without affecting the low molecular weight heparins, did not affect Factor H-mediated pneumococcal adherence (Fig. 1*A*). Immunofluorescence microscopy of adherent pneumococci confirmed the inhibitory effect of heparin and DS (Fig. 1*B*).

Binding of Pneumococcus-bound Factor H to Glycosaminoglycans Interferes with Bacterial Invasion into Epithelial Cells—In addition to impairing pneumococcal adherence, both heparin and DS significantly reduced Factor H-initiated pneumococcal invasion into epithelial cells (Fig. 1, *C* and *D*). Again, treatment of epithelial cells with heparinase III had, similar to adherence, no effect on pneumococcal invasion (Fig. 1*D*). These data imply that heparin and DS interfered with Factor H-mediated pneumococcal invasion into epithelial cells. How-

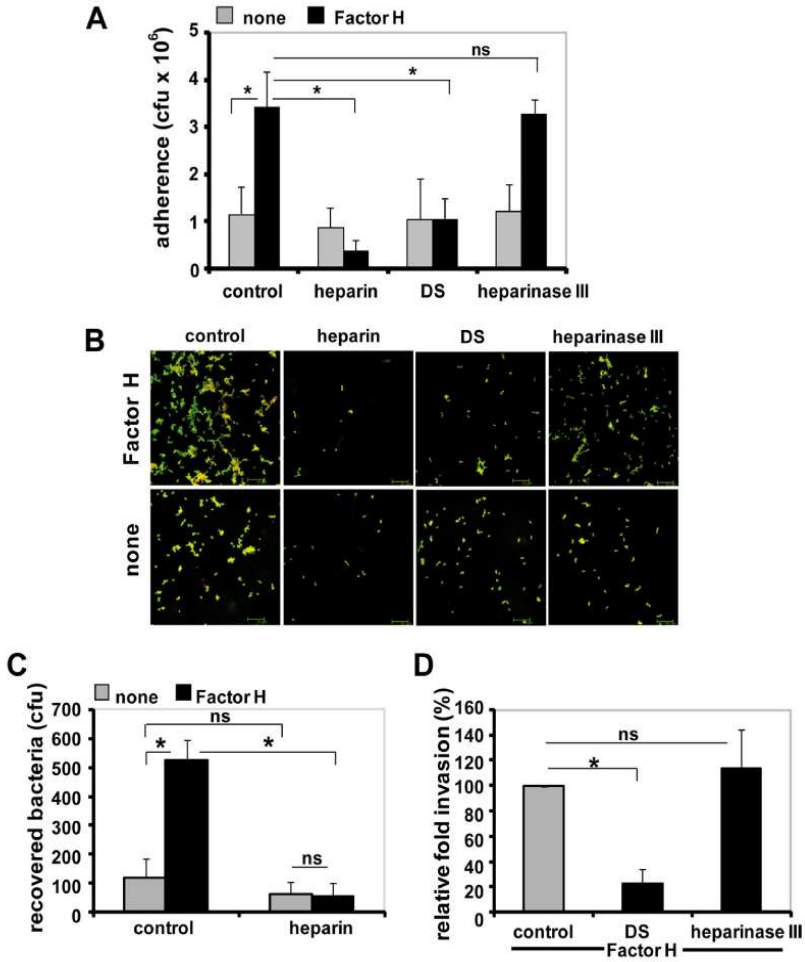

FIGURE 1. **Glycosaminoglycans inhibit Factor H-mediated pneumococcal adhesion to human lung epithelial cells.** *A*, adherence of pneumococci via Factor H to lung epithelial A549 cells in the absence (*control*) or the presence of heparin (50 units/ml), dermatan sulfate (100 μg/ml), or after pretreatment with heparinase III (10 milliunits/ml) was estimated by quantifying the colony-forming units (*cfu*) per well obtained from plating onto blood agar plates. The infection assays were conducted with or without (*none*) pretreatment of pneumococci with Factor H. *, $p < 0.02$. *B*, immunofluorescence microscopy of Factor H-mediated pneumococcal adhesion after treatment of host cells with glycosaminoglycans or heparinase III. Adherent bacteria appear *green/yellow* (Alexa Fluor 488/568), and intracellular bacteria were stained *red* (Alexa Fluor 568). *C* and *D*, pneumococcal invasion into epithelial cells via Factor H is diminished in the presence of glycosaminoglycans. Pneumococcal invasion was determined after conducting infection assays in the absence (*control*) or presence of glycosaminoglycans by employing the antibiotic protection assay. *, $p < 0.001$ relative to infections carried out with Factor H but in the absence of inhibitor; *ns*, not significant.

ever, this effect is not directly linked with invasion as the degree of reduction is the consequence of the diminished ability of pneumococci to adhere to epithelial cells when glycosaminoglycans were added to the infection assays.

SCR19–20 of Pneumococcal-bound Factor H Mediates the Contact with Host Epithelial Cells—To identify the region or SCR of Factor H that mediates pneumococcal attachment to the surface of eukaryotic cells, domain mapped mAbs for specific regions of Factor H were used as blocking antibodies. The mAbs CO2 and C18 employed as competitors bind within the C terminus of Factor H to SCR19 and SCR19–20, respectively (43, 44). The presence of CO2 and C18 caused 42 and 85%, respectively, reduction of Factor H-mediated pneumococcal adherence to A549 epithelial cells compared with bacterial adherence in the absence of competitors but presence of Factor H (Fig. 2*A*). In contrast, mAb M14, which binds to the middle region of Factor H, showed no effect (Fig. 2*A*). None of the three mAbs, however, affected the basal level of pneumococcal adherence in the absence of Factor H. Similar to adherence, the C-terminal binding mAbs CO2 and C18 reduced Factor H-mediated pneumococcal invasion into epithelial cells (Fig. 2*B*). In conclusion, the heparin-binding site at the C terminus of Factor H, namely SCR19–20, mediates the contact between pneumococcus-bound Factor H and the surface of epithelial cells. By blocking the C-terminal domain of Factor H attached to pneumococci, Factor H-mediated bacterial adherence to human epithelial cells is reduced. Consequently, pneumococcal ingestion by epithelial cells is also reduced to basal levels.

Acquisition of Factor H by Pneumococci Is Not Influenced by Soluble Heparin—The effect of heparin on the interaction between pneumococci and Factor H was analyzed in competitive binding experiments to elucidate whether heparin competes with Factor H for binding to the bacteria. Binding of FITC-labeled heparin was investigated to untreated and Factor H-pretreated pneumococci, respectively. The flow cytometric analyses showed no binding of FITC-heparin to the surface of untreated pneumococci, whereas FITC-heparin bound to Factor H-pretreated and consequently Factor H-coated pneumococci (Fig. 3*A*). Importantly, Factor H acquisition by pneumococci was not affected by heparin (Fig. 3*B*). However, when Factor H is bound to the pneumococcal cell surface, heparin binding is enhanced as it binds to the accessible heparin-binding sites of Factor H (Fig. 3*A*). In conclusion, the presence of heparin did not interfere with pneumococcal acquisition of Factor H but blocks the C-terminal heparin-binding site of Factor H, which is involved in Factor H-mediated adherence.

Factor H Promotes Pneumococcal Binding to PMNs via Integrin CR3—Binding of Factor H by pneumococci resulted in reduced complement activation on the pneumococcal surface and diminished complement-mediated opsonophagocytosis (8,

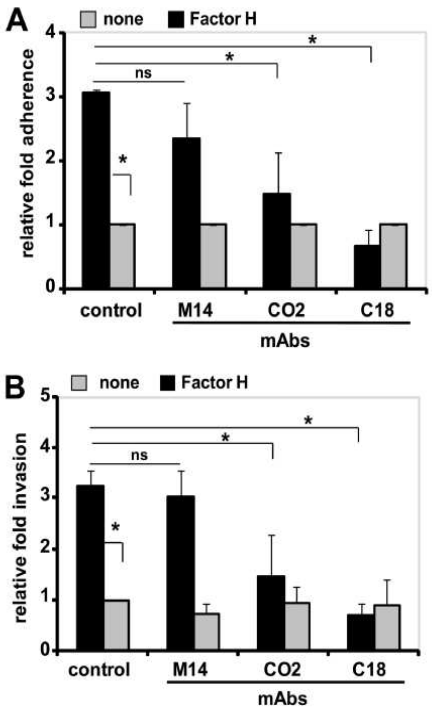

FIGURE 2. **C-terminal SCR19–20 of Factor H is the key co-factor in Factor H-mediated pneumococcal adherence to and invasion into host cells.** *A*, adherence of Factor H-coated pneumococci to A549 cells was determined in the presence of mAbs M14, CO2, or C18 (each 2 μg ml^{-1} per well) or absence of a mAb (*control*). The antibodies bind to the middle region (*M14*), SCR19 (*CO2*), or SCR19–20 (*C18*) of Factor H. Results are shown as relative adherence of Factor H-bound pneumococci compared with untreated pneumococci. *, $p < 0.02$; *ns*, not significant. *B*, invasion and intracellular survival of Factor H-coated pneumococci in the presence of monoclonal antibodies M14, CO2, or C18 (each 2 μg ml^{-1}) were determined by the antibiotic protection assay. Results show the relative invasion of pneumococci into A549 cells compared with Factor H-treated bacteria and in the absence (*control*) of monoclonal antibodies. *, $p < 0.02$; *ns*, not significant.

13, 14). As Factor H binds to the integrin CR3 of PMNs (37), we assessed whether CR3 acts as a receptor on PMNs and thereby promotes adhesion of Factor H-coated pneumococci. Pretreatment of pneumococci with Factor H resulted in increased association of pneumococci with PMNs as determined by flow cytometry (Fig. 4). However, mAbs that block specifically CD11b and CD18, respectively, and also the CR3 ligand Pra1 (pH-regulated antigen 1) of *Candida albicans* (45) abolished binding of Factor H-coated pneumococci to human PMNs (Fig. 4). In contrast, the corresponding isotype control antibodies IgG1 and IgG2, respectively, showed no effect (data not shown). These data suggest that integrin CR3 of PMNs recognizes Factor H-coated pneumococci and that Factor H bound to pneumococci promotes pneumococcal binding to PMNs.

CR3 Facilitates Factor H-mediated Pneumococcal Invasion into Eukaryotic Cells—To assess the role of CR3 as host cell receptor facilitating ingestion of Factor H-coated pneumococci by epithelial cells, CHO cells expressing human CR3 (42) were infected with Factor H-coated pneumococci; thereafter, bacterial invasion was assayed and compared with wild-type CHO cells lacking the CR3 receptor. Invasion of Factor H-coated pneumococci into CR3-expressing CHO cells was >2-fold increased compared with untreated pneumococci. However, invasion of Factor H-coated pneumococci into wild-type CHO-K1 cells that lack CR3 was also increased but at a relatively low level, and the Factor H effect in wild-type CHO cells was statistically not significant (Fig. 5*A*). Adherence of pneumococci to CR3-expressing and wild-type CHO cells was similar as visualized by immunofluorescence microscopy (data not shown). However, adherence of pneumococci to CHO-CR3 cells via Factor H was significantly decreased when blocking antibodies to the subunits of CR3, namely CD11b and CD18, respectively, or Pra1 was used as a competitive inhibitor in the infections (data not shown).

Factor H facilitates pneumococcal invasion into human lung epithelial cells A549 and also into CHO-K1 wild-type cells, although the latter at relatively low levels (Figs. 2 and 5). Consequently, we assumed the existence of an additional receptor on epithelial cells that may contribute to Factor H-mediated pneumococcal uptake by these host cells. Moreover, A549 lung epithelial cells are not known to express the CR3 receptor (51). Therefore, the fungal Pra1 protein, which is a CR3 ligand and which, however, binds also to non-CR3-expressing endothelial cells,[4] was used as a competitor to investigate whether Pra1 can also inhibit infections of Factor H-coated pneumococci to A549 cells. Pra1 did not affect Factor H-mediated pneumococcal adhesion to A549 epithelial cells (Fig. 6, *A* and *B*). However, Pra1 inhibited pneumococcal invasion of lung epithelial cells via the Factor H mechanism by ~45% (Fig. 6*C*). Taken together, pneumococcal adherence via Factor H is mediated by heparin and DS, and bacterial invasion into host cells is receptor-mediated. Moreover, Factor H-mediated invasion can be blocked efficiently by the integrin-ligand Pra1.

Actin Cytoskeleton Dynamics Are Essential for Factor H-mediated Pneumococcal Internalization by Epithelial Cells—The impact of the actin cytoskeleton and microtubules on pneumococcal internalization by host cells via bacterially bound Factor H was investigated by employing the pharmacological inhibitors cytochalasin D and nocodazole. Cytochalasin D, which inhibits actin polymerization, inhibited significantly Factor H-mediated pneumococcal invasion into A549 cells indicating the important role of the actin dynamics for pneumococcal uptake (Fig. 7*A*). In contrast, inhibition of microtubule polymerization by nocodazole did not affect internalization of Factor H-coated pneumococci by host cells (Fig. 7*B*).

Factor H-mediated Pneumococcal Invasion Relies on Protein-Tyrosine Kinase and PI3K Activities—Protein-tyrosine kinases, PI3K, and the activities of the small GTPases of Rho family are essential for uptake of various pathogenic bacteria by host cells. Phosphatidylinositol 3-kinase activity is critical for the invasion of host cells by pathogenic bacteria such as *Listeria monocytogenes*, *Helicobacter pylori*, and *Escherichia coli* (52–54). In addition, the small GTPase RhoA has been demonstrated to be important for uptake of *Mycobacterium avium* and *Pseudomonas aeruginosa* (55, 56), whereas Rac1 and Cdc42 play a crucial role in host cell invasion of *Salmonella enterica*, *Shigella flex-*

[4] S. Luo and P. F. Zipfel, manuscript in preparation.

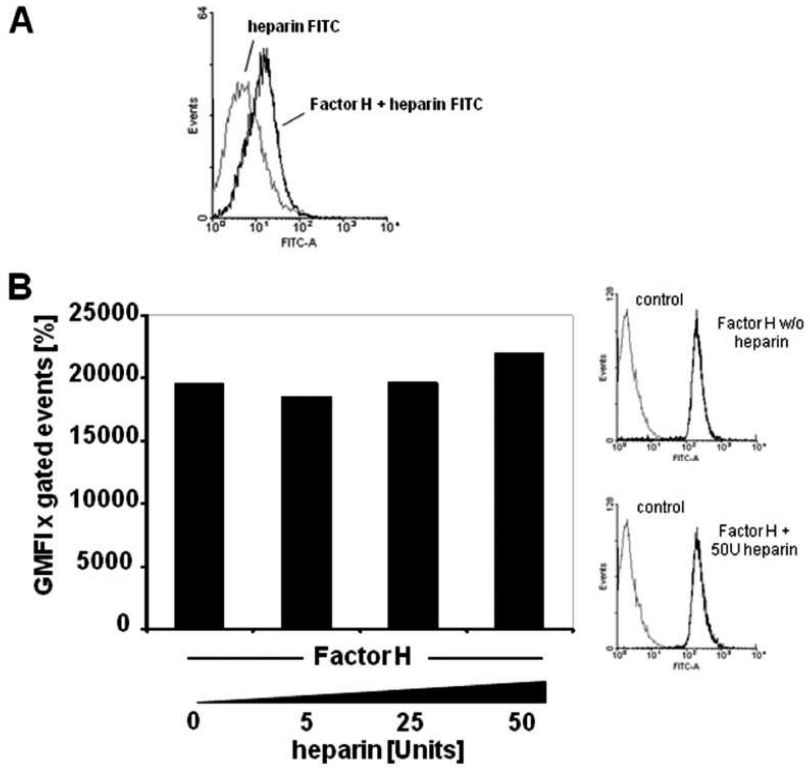

FIGURE 3. **Effect of heparin on Factor H recruitment by pneumococci.** *A*, binding of FITC-heparin (2 μg) to pneumococci or to Factor H-pretreated pneumococci was determined by flow cytometry. The *histogram* shows the log fluorescence intensity (*FITC-A*) on the *x* axis, and the *y* axis shows the number of events. *B*, binding of heparin-pretreated Factor H to pneumococci. The effect of heparin on pneumococcal recruitment of Factor H was analyzed by flow cytometry after preincubation of 2 μg of purified Factor H with the indicated amounts of heparin per reaction. Bacterial bound Factor H was determined by flow cytometry, and results were expressed as mean fluorescence intensity multiplied with the percentage of FITC-labeled bacteria. The *graph* shows a representative experiment. The results are also represented as *histograms*, where the *x* axis represents fluorescence of pneumococcus-associated Factor H, in the absence (*w/o*) or presence of 50 units of heparin, on a $\log_{10}$ scale, and the *y* axis represents the number of events.

neri, and *Campylobacter jejuni* (57–59). Finally, Rho family GTPases RhoA, Rac1, and Cdc42 are required for efficient invasion of HeLa cells by group B streptococci (60).

To explore the role of protein-tyrosine kinases and PI3K for ingestion of Factor H-coated pneumococci by epithelial cells, bacterial invasion into A549 cells was determined in the presence of protein-tyrosine kinase inhibitors genistein or PI3K-specific inhibitor wortmannin. The results revealed that both inhibitors blocked Factor H-mediated pneumococcal internalization in a dose-dependent manner (Fig. 8, *A* and *B*). To elucidate the role of small GTPases (RhoA, Rac1, and Cdc42), *C. difficile* toxin B TcdB10463, which glucosylates and inactivates the Rho family of small GTPases Rho (A/B/C), Rac1, and Cdc42, was employed. In addition, Y27632, a specific Rho-associated protein kinase inhibitor, NSC23766, a specific Rac1 inhibitor, or secramine A, which is a potent inhibitor of Cdc42 activation, were also used. Pretreatment of A549 cells with TcdB10463 did not affect Factor H-mediated pneumococcal uptake by host cells (Fig. 8*C*). Similarly, treatment of the host cells with Y27632 or NSC23766 did not affect pneumococcal invasion (Fig. 8*D*). Although secramine A pretreatment of A549 cells resulted in significant reduction in Factor H-initiated pneumococcal invasion, a similar level of reduction was also observed for Factor H untreated pneumococci (Fig. 8*D*), suggesting that Cdc42 is not involved in pneumococcal invasion via Factor H. The concentration of the inhibitors used did not affect the increase in Factor H-mediated pneumococcal adherence (data not shown). Taken together, the data indicated the essential roles of host cell protein-tyrosine kinases and PI3Ks for invasion of Factor H-coated pneumococci.

DISCUSSION

The complement system is part of the host innate immune system and pivotal for the host defense mechanisms. To establish an infection, the pathogen has to overcome this first line of immune defense. Hence, pathogens have evolved various efficient immune evasion strategies to counteract host complement attacks (61–65). A strategy that has attracted particular interest is the ability of pathogenic microorganisms to acquire fluid phase soluble complement regulators to their surface. Factor H, a fluid phase regulator of the alternative complement pathway, is a central host protein that is acquired by pathogens and attached to the pathogen surface it aids in immune evasion and avoids complement-mediated killing. Pneumococci, serious pathogenic bacteria causing life-threatening infections in humans, recruit Factor H to their surface (9–15). Recently, we demonstrated that Factor H bound to pneumococci via an interaction with PspC increased in a cell type unspecific manner bacterial attachment to and invasion into host cells (17). Here, we demonstrate that Factor H-promoted pneumococcal invasion is at least a two-step process that requires a concerted action of host epithelial cell surface glycosaminoglycans, integrin receptors, as well as host cell-signaling molecules of different pathways. Moreover, the interaction of bacterial bound Factor H with glycosaminoglycans and integrins suggests a critical role for the C terminus of the protein, *i.e.* SCR19–20, as well as the RGD-containing SCR4 of Factor H that may interact with the integrins for the infectious process (Fig. 9).

Factor H is an abundant human plasma protein that binds to the surface of host cells and other self-surface moieties via recognition of polyanionic components such as glycosaminogly-

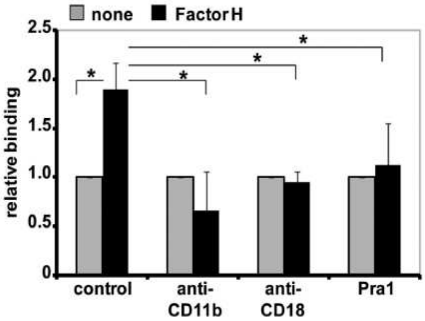

FIGURE 4. **Factor H promotes pneumococcal binding to PMNs via integrin CR3.** Pneumococci were incubated with PMNs for 30 min, in the absence (*control*) or presence of anti-CD11b (2 μg), anti-CD18 (2 μg), or integrin CR3 (CD11b/CD18) ligand Pra1 (2 μg). Pneumococcal binding to PMNs was investigated in the absence (*none*) or presence of bacteria-bound Factor H by flow cytometry. The results were calculated (mean fluorescence intensity × percentage of gated positive events), and the data show the relative binding ratios compared with Factor H untreated pneumococci. *, $p < 0.02$.

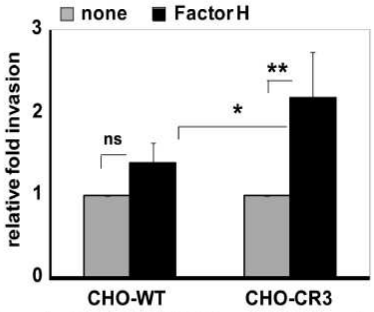

FIGURE 5. **Integrin CR3 (CD11b/CD18) promotes invasion of Factor H-coated pneumococci into epithelial cells.** Invasion and intracellular survival of pneumococci in CHO-WT (CHO-K1 cells) and CHO-CR3 cells were determined by the antibiotic protection assay. The results are shown relative to infections conducted with untreated pneumococci. *, $p < 0.05$; **, $p < 0.01$; *ns*, not significant.

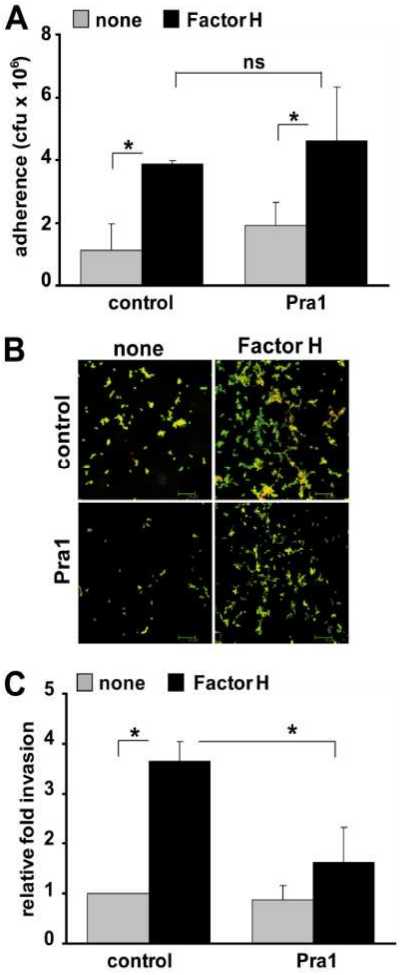

FIGURE 6. **Factor H-mediated pneumococcal internalization by epithelial cells is inhibited by the integrin-binding protein Pra1.** *A*, pneumococcal adherence to A549 cells was determined in the absence (*control*) or presence of integrin CR3 ligand Pra1 (2 μg ml^{-1}). The infection assays were conducted with or without (*none*) pretreatment of pneumococci with Factor H. *, $p < 0.03$; *ns*, not significant, relative to infections conducted in the absence of Factor H. *B*, immunofluorescence microscopy of pneumococcal adherence via Factor H to A549 cells in the absence (*control*) or presence of Pra1. *C*, invasion and intracellular survival of pneumococci in A549 cells were monitored in the absence (*control*) or the presence of Pra1 (2 μg ml^{-1}) by the antibiotic protection assay. The results are shown relative to infections conducted in the absence of Pra1 and Factor H. *, $p < 0.02$.

cans and sialic acids (24, 26). The cell surface of the used A549 lung epithelial cells is decorated with polyanionic components such as heparan sulfate, dermatan sulfate, or chondroitin sulfate (41). The impact of host cell glycosaminoglycans for adherence of Factor H-coated pneumococci was demonstrated in inhibition experiments with heparin or dermatan sulfate as competitors. Both glycosaminoglycans significantly reduced adherence and also invasion of Factor H-coated pneumococci into human epithelial cells, whereas treatment of host cells with heparinase III, an enzyme that cleaves exclusively heparan sulfate on cell surfaces, showed no inhibitory effect. This is in contrast to pneumococcal adherence via human thrombospondin-1, which was significantly reduced by host cell treatment with heparinase III (39). Taken together, these data point to a critical role of the heparin/glycosaminoglycan-binding sites within the Factor H molecule. In addition, the competitive binding assays demonstrated that heparin did not compete with Factor H for binding to pneumococci, whereas it can interact with Factor H attached to the pneumococcal surface. These results seem not to be in agreement with the *in vitro* effect of heparin to block Factor H binding to PspC protein as shown by surface plasmon resonance studies (17). However, the assays conducted here represent a closer *in vivo* scenario. Under physiological conditions, the SCR8–11 of Factor H, which acts as the major binding region for PspC and lacks a heparin-binding site, represent the binding region required for recruitment of Factor H to the pneumococcal surface. The major heparin/glycosaminoglycan-binding site of Factor H is located in the C terminus, in SCR19–20, and mediates binding of Factor H to endothelial cells (43, 66, 67). This region is also pivotal and makes the first contact of pneumococci to host cells as only mAbs, which bind to this particular region, block Factor H-mediated bacterial adherence to host cells. In contrast, mAbs that bind to the middle region of Factor H had no effect.

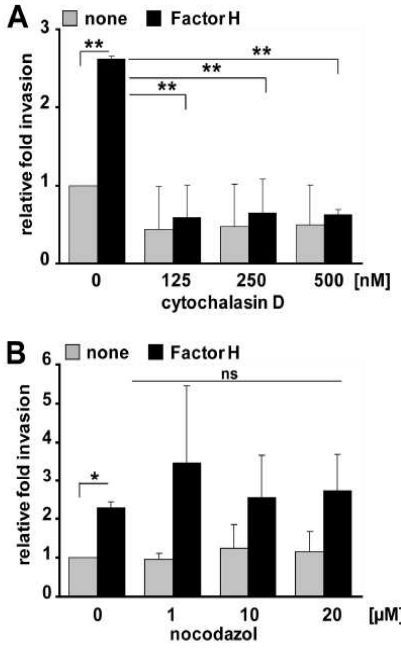

FIGURE 7. **Factor H-mediated pneumococcal ingestion by epithelial cells requires actin cytoskeleton dynamics.** *A* and *B*, pneumococcal invasion into A549 was determined in the presence of cytochalasin D (*A*) or nocodazole (*B*) by the antibiotic protection assay as described previously (47). Pneumococcal invasion indicates the relative number of intracellular bacteria (Factor H pretreated or untreated (*none*)) in the presence of an inhibitor compared with pneumococcal invasion in the absence of an inhibitor. *, $p < 0.05$; **, $p < 0.001$; *ns*, not significant.

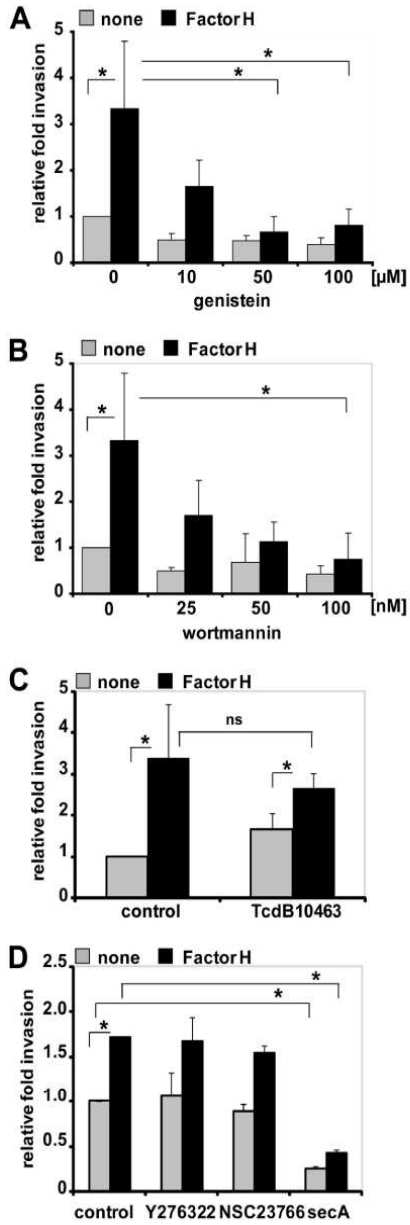

FIGURE 8. **Protein-tyrosine kinase activities and PI3K but not small Rho family GTPases are essential for Factor H-mediated pneumococcal ingestion by epithelial cells.** The number of invasive pneumococci was determined in the presence of genistein (*A*), which is a phosphotyrosine kinase inhibitor, PI3K inhibitor wortmannin (*B*), *C. difficile* toxin B, TcdB10463 (30 ng ml^{-1}) (*C*), or specific individual inhibitors of Rho family GTPases such as Y27632 (50 μM), Rac1 inhibitor NSC23766 (50 μM), or Cdc42 inhibitor secramine A (10 μM) (*D*) by employing the antibiotic protection assay. Shown is the relative invasion of Factor H pretreated or untreated (*none*) pneumococci in the presence of an inhibitor compared with pneumococcal invasion in the absence of an inhibitor. *, $p < 0.05$; *ns*, not significant, relative to infections in the presence of Factor H but absence of inhibitors.

Factor H is also an adhesion molecule for human PMNs, and the integrin CR3 was identified as its cellular receptor on PMNs (37). Moreover, adherence of PMNs to immobilized glycosaminoglycans such as heparin and chondroitin was enhanced in the presence of Factor H, suggesting that in this scenario Factor H promotes adhesion of PMNs to tissues (37). Therefore, we investigated the effect of Factor H on pneumococcal attachment to PMNs and aimed to identify the receptor(s) engaged by pneumococcus-bound Factor H. Apparently, Factor H-enhanced attachment of pneumococci to PMNs and CR3 was identified as a cellular receptor required for augmented bacterial attachment and probably also for enhanced phagocytosis. Inhibition experiments conducted with mAbs blocking each subunit of the dimeric CR3 receptor (*i.e.* anti-CD11b and anti-CD18) and also the fungal CR3-ligand Pra1 of *C. albicans* (45) confirmed an essential role of this cellular receptor for attachment of Factor H-coated pneumococci. Infection assays performed with CHO cells expressing CR3 further demonstrated that this integrin can also act as a cellular receptor facilitating Factor H-dependent invasion of pneumococci into epithelial cells. Moreover, pneumococcus-bound Factor H promoted bacterial invasion into A549 cells, and this effect was dramatically blocked by Pra1. Strikingly, Pra1 had no inhibitory effect on the increased adherence of Factor H-coated pneumococci to lung epithelial cells, whereas a significant reduction was observed when using CHO-CR3 cells (data now shown). These results suggested that host cells use a different repertoire of Factor H receptors and also that the Pra1 protein interacts with several distinct receptors expressed on the surface of human cells that allow Factor H-mediated uptake of pneumococci by

Mechanisms of Factor H-mediated Pneumococcal Invasion

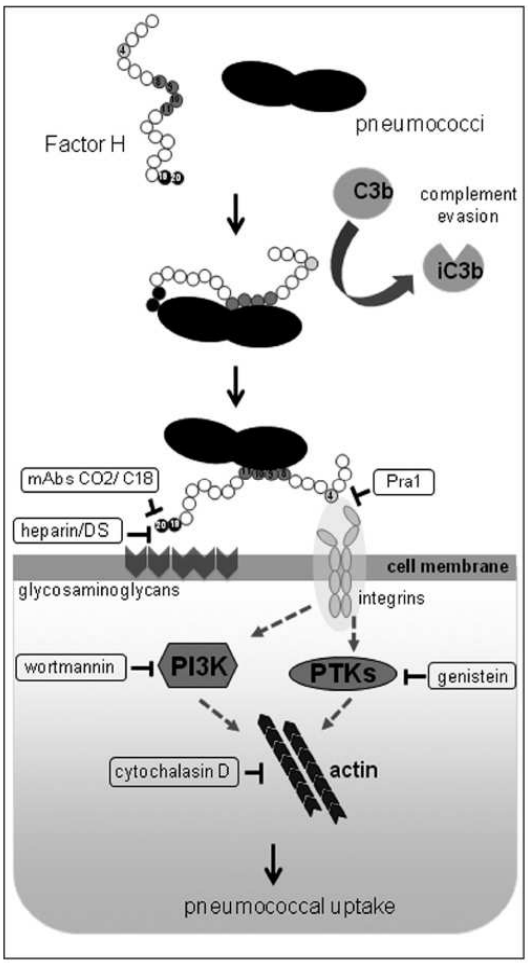

FIGURE 9. **Schematic model of the two-step Factor H-integrin complex-mediated pneumococcal epithelial cell invasion mechanism.** *PTK,* protein-tyrosine kinase.

host cells. Similar to adhesive glycoproteins such as fibronectin, the Factor H molecule contains in SCR4 an Arg-Gly-Asp sequence. These tripeptides bind to different integrin receptors as has been shown for fibronectin, for example (68). Factor H binding to CR3 and its likely interaction via Arg-Gly-Asp to an additional integrin receptor suggest that integrin(s) acts as receptors and mediators of Factor H-promoted pneumococcal and bacterial invasion. This hypothesis is strengthened by the fact that the Pra1 protein binds directly to CR3 and also to an additional cellular receptor as Pra1 inhibited Factor H-mediated invasion of pneumococci into A549 cells.

This study demonstrates the importance and the impact of host cell cytoskeleton and signaling molecules for the Factor H-mediated bacterial internalization into eukaryotic cells. This Factor H mechanism requires the dynamics of the actin cytoskeleton but not microtubules, and this effect is in contrast to PspC-pIgR-mediated pneumococcal internalization into human cells as shown recently (47). Cytochalasin D significantly inhibited Factor H-mediated pneumococcal invasion into human lung epithelial cells, and inhibition of microtubule polymerization did not interfere with Factor H-dependent pneumococcal uptake. Moreover, inhibition assays showed that the activities of protein-tyrosine kinases and PI3K but not small GTPases of the Rho family (RhoA, Rac1, and Cdc42) are essential for Factor H-mediated pneumococcal ingestion by host epithelial cells. Pretreatment of A549 lung epithelial cells with genistein, which is a general protein-tyrosine kinase inhibitor, or with wortmannin, a specific PI3K inhibitor, blocked pneumococcal internalization in a dose-dependent manner. These effects are similar to another integrin-mediated pneumococcal uptake mechanism (38). However, in contrast to PspC-pIgR-mediated pneumococcal uptake that requires the activity of small GTPase Cdc42, pneumococcal internalization via the bridging molecule Factor H does not rely on small GTPases. Inhibition of endogenous Rho family members by *C. difficile* toxin B TcdB-10463 did not affect Factor H-dependent pneumococcal uptake by host cells. Similarly, specific inhibition of Rac1 using NSC23766 or blocking of Rho-associated protein kinase using the inhibitory substance Y27632 had no effect on Factor H-coated pneumococcal uptake by host cells. Apparently, inhibition of Cdc42 activity by secramine A resulted in a significant reduction of pneumococcal uptake by Factor H mechanism. Similar, secramine A inhibited invasion of Factor H-untreated pneumococci, suggesting that Cdc42 also plays no significant role in the Factor H-mediated pneumococcal invasion mechanism.

Further investigations are required to identify the individual kinases that are activated during Factor H-mediated pneumococcal uptake by host cells and to delineate the outside-inside and inside-outside signaling events during Factor H-mediated pneumococcal infection of eukaryotic host cells.

In conclusion, Factor H acquisition by pneumococci endows the bacteria with an adhesive host-derived molecule that on mucosal epithelial surfaces promotes pneumococcal adherence to and invasion into host cells. This two-step mechanism requires a concerted action of glycosaminoglycans expressed on the surface of host epithelial cells and of integrin receptors and also host cell signaling proteins and pathways.

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5. Discussion

Function of Pra1 as a surface protein of *C. albicans*

Surface Pra1 binds the human complement regulators Factor H, FHL-1, C4BP as well as plasminogen

Acquisition of human complement regulators is a general immune evasion strategy used by *C. albicans*, as well as many other pathogens, such as *A. fumingutas* and *S. pneumoniae* [99,100,103,230]. *C. albicans* acquires human complement regulators Factor H, FHL-1, and C4BP to the fungal surface and utilizes these regulators for complement evasion [99,100]. Through screening a cDNA library, Pra1 was identified as a Factor H binding protein. Pra1 is a strain-specific glycosylated protein composed of 299 amino acids [193,234,237,238], which has several consensus sites for N-linked as well as O-linked glycosylation. Based on the glycosylation, treatment of the native Pra1 protein with endoglycosidase H reduced the molecular mass by ca. 25% [237]. Using the specific anti-serum, I localized Pra1 at the surface of both the yeast and hyphal forms of *C. albicans* and showed that Pra1 is also released into the culture supernatant which is consistent with the previous study [234,235,237]. As a surface protein, Pra1 is primarily localized at the tip of *C. albicans* hyphae, suggesting an important role of Pra1 upon contact with host tissues and surfaces during infection (**manuscript 1**).

Pra1, also termed *Candida* CRASP2 (*complement regulator acquiring surface protein 2*), is a novel Factor H and FHL-1 binding fungal surface protein identified from human pathogenic yeast *C. albicans*. Pra1 binds the human complement regulator Factor H, via N-terminal SCRs5-7 and also C-terminal SCRs16-20, and binds FHL-1 via SCRs5-7. This binding property is similar, but not identical to *Candida* moonlighting protein phosphoglycerate mutase 1 (Gpm1p), also termed *Candida* CRASP1. Gpm1p binds Factor H via SCRs6-7 and SCRs19-20, and binds FHL-1 via SCRs6-7 [101]. The interaction between Pra1 and Factor H is affected by NaCl, which implies that this binding is mostly based on ionic interaction between amino acids (**manuscript 1**).

A Pra1 specific anti-serum inhibits binding of Factor H and FHL-1 to *C. albicans* by ca. 17% and 40%, respectively (**manuscript 1**), which indicates that native Pra1 at the surface of the yeast binds Factor H and FHL-1. The relative low level of reduction is explained by the existence of additional Factor H and FHL-1 binding proteins expressed by *C. albicans.* For example, *Candida* Gpm1 is an additional Factor H/FHL-1 binding protein of *C. albicans* [101].

In this work, Pra1 was also identified as the first fungal C4BP binding protein from *C. albicans*. *Candida* Pra1 binds both serum derived and recombinant C4BP. The Pra1-C4BP interaction is of ionic interaction and is affected by NaCl. This type of interaction is similar to the Pra1-Factor H interaction, but is different from that of the streptococcal M proteins-C4BP interaction, which is of hydrophobic nature [137]. *Candida* Pra1 binds C4BP via domains SCR4, SCR7 and SCR8, which is different from the complement inhibitory activity sites, SCRs1-3 [56,239]. Thereby, binding of Pra1 to C4BP via C-terminal domains SCR4, 7 and 8 orientates C4BP in such a way that the protein better exposes the functional regions to the outside. Rather similar attachment of other microbial proteins to C4BP has been defined for *ubiquitous surface protein A 1* (UspA 1) and UspA 2 of *M. catarrhalis,* that bind C4BP via domains SCR2 and SCR7 [122], outer *membrane protein A* (OmpA) of *Escherichia coli* K1 and *Haemophilus influenzae* [118] that bind C4BP via domains SCR3 and SCR8 [116]. However, streptococcal M protein [137] and *Neisseria gonorrhoeae* porins [240] bind to C4BP via the N-terminal SCR1. Filamentous hemagglutinin of *Bordetella pertussis* [241] bind to C4BP via SCR1 and SCR2. Similarly when analyzing intact *C. albicans*, the relevant binding domains within C4BP were localized to SCR1 and SCR2 for both yeast and hyphal forms [100]. This difference between C4BP binding to Pra1 and to intact *C. albicans* cells suggests that the existence of an additional C4BP ligand(s) expressed at the surface of *C. albicans*.

In addition, Pra1 was identified as a new fungal plasminogen binding protein (**manuscript 1**). Pra1-plasminogen interaction is also inhibited by NaCl and is mostly based on ionic interaction. However, this effect is not as much pronounced as the Pra1-Factor H interaction. In addition, the inhibition of lysine analogue aminocaproic acid on Pra1-plasminogen interaction suggests that lysine residues mediate the contact of Pra1 with human plasminogen. A Pra1 specific anti-serum inhibits plasminogen binding to *C. albicans* surface by ca. 12% (**manuscript 1**), which confirms the role of surface Pra1 in mediating *Candida*-plasminogen binding. However, this low level of reduction indicates that additional plasminogen binding proteins exist at the *C. albicans* surface. By a proteomic approach, eight plasminogen binding proteins were identified, including alcohol dehydrogenase, thioredoxin peroxidase, catalase, transcription elongation factor, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, Gpm1 and fructose bisphosphate aldolase [101,212].

A Pra1 overexpressing *C. albicans* strain, which has about two fold Pra1 levels at *C. albicans* surface binds Factor H, C4BP and plasminogen more efficiently, as compared to the wild type

strain (**manuscript 1 and 3**). However, a Pra1 knock out mutant shows 9%, 22% and 30% reduction for Factor H, C4BP and plasminogen binding, respectively, as compared to the wild type strain. These binding properties of Pra1 mutants indicate that surface expressed, native Pra1 acquires human complement regulators Factor H, C4BP as well as plasminogen from the serum to the surface of *C. albicans* for complement evasion, and that additional Factor H, C4BP, as well as plasminogen binding proteins exist at the surface of *C. albicans* (**manuscript 3**).

C4BP and Factor H binding to Pra1 is not affected by the lysine analogue, while plasminogen binding to Pra1 is. This suggests that Factor H and C4BP do not compete with plasminogen for Pra1 binding. In addition, C4BP does not compete with Factor H for binding to Pra1 when C4BP is less than five times of Factor H amount (**manuscript 3**), which indicates that Factor H and C4BP also bind independently to Pra1 at the physiological mass ratio in human plasma (C4BP, ca. 200 µg/ml and Factor H, ca. 500 µg/ml). Thus, human complement regulator Factor H, FHL-1, C4BP as well as plasminogen bind to surface Pra1 at non-overlapped contact sites.

C. albicans utilizes surface Pra1 for complement evasion and tissue invasion

C. albicans yeast and hyphae use Pra1 to acquire human complement regulators Factor H, FHL-1 and C4BP to the surface. Attached to Pra1, each human regulator is functionally active. Factor H, FHL-1 and C4BP bound to Pra1 maintain their cofactor activity and assist Factor I to degrade C3b or C4b. Consequently C3b and C4b inactivation will inhibit the formation of C3 convertases (C3bBb and C4bC2b) of both the alternative and the classical pathways on the fungal surface. This likely prevents further progression of the complement cascade, thereby inhibiting down-stream immune effector functions, like phagocytosis, inflammation and lysis of microbes. In addition, plasminogen bound to Pra1 is accessible to the activator uPA and is converted to the serine protease plasmin. The activated plasmin degrades both the synthetic chromogenic substrate S2251 and the native extra-cellular matrix component fibrinogen (**manuscript 1**), thereby aiding *C. albicans* in tissue invasion. In summary, surface expressed Pra1 by binding to human complement inhibitors Factor H, FHL-1 and C4BP, mediates *C. albicans* in complement evasion, and by binding to human plasminogen, mediates the fungal tissue invasion. (**Figure 9**).

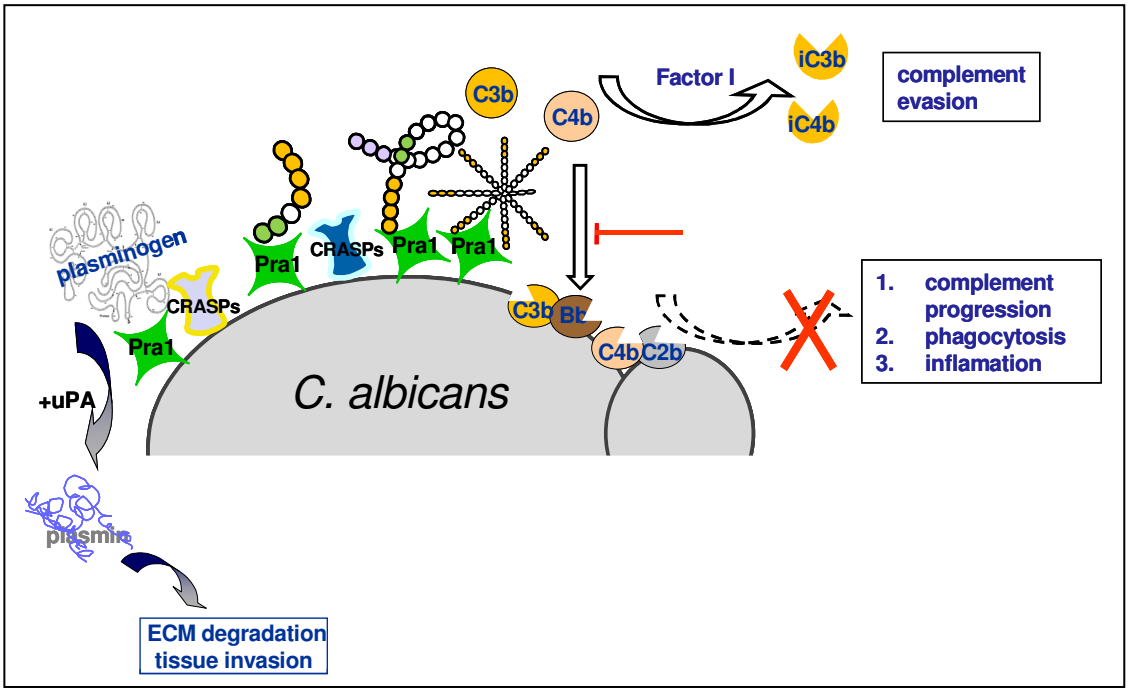

Figure 9. The model of Pra1 mediated complement evasion and tissue invasion of *C. albicans*. *C. albicans* utilizes surface proteins, like Pra1 and other CRASPs to acquire human complement regulators Factor H, FHL-1 and C4BP to the surface for immune and complement evasion and binds plasminogen for tissue invasion.

Function of Pra1 as a released protein of *C. albicans*

Soluble Pra1 is a potent complement inhibitor

Pra1 is secreted into the culture supernatant by both yeast and hyphal forms of *C. albicans*. Soluble Pra1 is newly discovered as a potent complement inhibitor, which is, -to our knowledge-, the first fungal complement inhibitor. Pra1 inhibits C3a generation in a dose dependent manner, which indicates that the inhibitory effect of Pra1 on complement activation occurs at the level of C3 convertase. Pra1 binds C3 and C3b. However Pra1 does neither inhibit C3 convertase formation, nor dissociate a preformed C3 convertase. Further characterization indicates that Pra1 displays its complement inhibitory effect in the fluid phase, but not on the surface (**manuscript 2**). Aiming for the inhibitory mechanism, I found that secreted Pra1, by complexing C3 in solution inhibits C3 cleavage by C3 convertases, consequently blocks complement progression, further formation of the amplification loop and the downstream effector functions, such as inflammation and phagocytosis (**Figure 10**). This complement inhibitory mechanism of Pra1 is similar or related to that of Efb-C from the Gram positive bacterium *S. aureus*, which also binds C3 and blocks C3 cleavage by the C3 convertases [88]. However, *Candida* Pra1 acts differently from *S. aureus* inhibitor SCIN which blocks complement activation by stabilizing C3 convertases [86]. In addition, secreted Pra1 also binds Factor H in fluid phase and enhances the complement regulatory activity of Factor H in

mediating cleavage of C3b into iC3b by Factor I (**manuscript 1**). Therefore, secreted Pra1 forms a double-protective layer against complement attack in the surrounding of *C. albicans*. Furthermore, secreted Pra1 binds back to the surface of *C. albicans* hyphae, and functions as a surface protein to acquire human complement regulators and plasminogen to mediate complement evasion and tissue invasion.

As a complement inhibitor, Pra1 also inhibits C5a generation. However, as Pra1 has already blocked complement activation at the level of C3, it is currently unclear whether the blockade of C5a release is a direct effect by inhibiting the assembly of the C5 convertase (C3bBbC3b or C4bC2bC3b), or an indirect effect due to the inhibition of C3 conversion. Independent of the exact mechanism, *Candida* Pra1 mediated inhibition of C3a- and C5a generation may prevent inflammatory effector functions, like recruitment of immune effector cells to the sites of infection [242,243]. Thus, secreted Pra1, by inhibiting complement activation, builds up an anti-inflammatory environment, which favors *C. albicans* survival.

Soluble Pra1 by blocking C3 conversion inhibits C3b/iC3b mediated adhesion and phagocytosis of the yeast by human macrophages

C3b or iC3b, when deposited onto the surface of a microbe, are efficiently recognized by complement receptors, i.e CR1 or CR3 which are expressed on the surface of human phagocytes, thus favoring phagocytosis and efficient removal of opsonized microbes [244]. As a complement inhibitor, Pra1 by complexing C3 in solution, blocks C3 conversion, thereby inhibiting C3b/iC3b generation and deposition on the surface of *C. albicans* upon either AP or CP/LP activation (**manuscript 2**). Consequently, this inhibitory effect interferes with C3b/iC3b mediated adhesion and phagocytosis of the yeast by human macrophages and aids in the immune escape of the yeast from cellular immune responses. However, the inhibition of Pra1 on C3b/iC3b mediated adhesion and phagocytosis is only partial because multiple other receptors also play a role for recognition of *C. albicans* by macrophages, like Toll-like receptors and dectin-1 [151,245,246].

The biological function of Pra1 correlates with the concentration of native secreted Pra1

Pra1 is secreted by *C. albicans* in a time dependent manner. Ten million yeast cells secrete ca. 5 µg of Pra1 within 24 h (**manuscript 2**). This concentration correlates with the biological effect observed for recombinant Pra1 (1~10 µg). However, during infection and tissue invasion, local Pra1 levels may even be higher, as Pra1 expression is highly up-regulated upon

hyphal induction [247]. This provides further evidence for the biological relevance of Pra1 *in vivo*.

Taken together, *C. albicans* secretes a potent complement inhibitor Pra1, which in the direct surrounding of the pathogen, (i) complexes C3 in fluid phase, (ii) inhibits C3 cleavage by C3 convertases of both the AP and the CP/LP, thereby blocking the release of the anaphylatoxins C3a and C5a, as well as C3b/iC3b surface deposition, (iii) by blocking C3 conversion, inhibits further complement progression and downstream effector function, such as anaphylotoxin C3a and C5a based inflammation, as well as C3b/iC3b mediated adhesion and phagocytosis of the yeast by human macrophages (**Figure 10**).

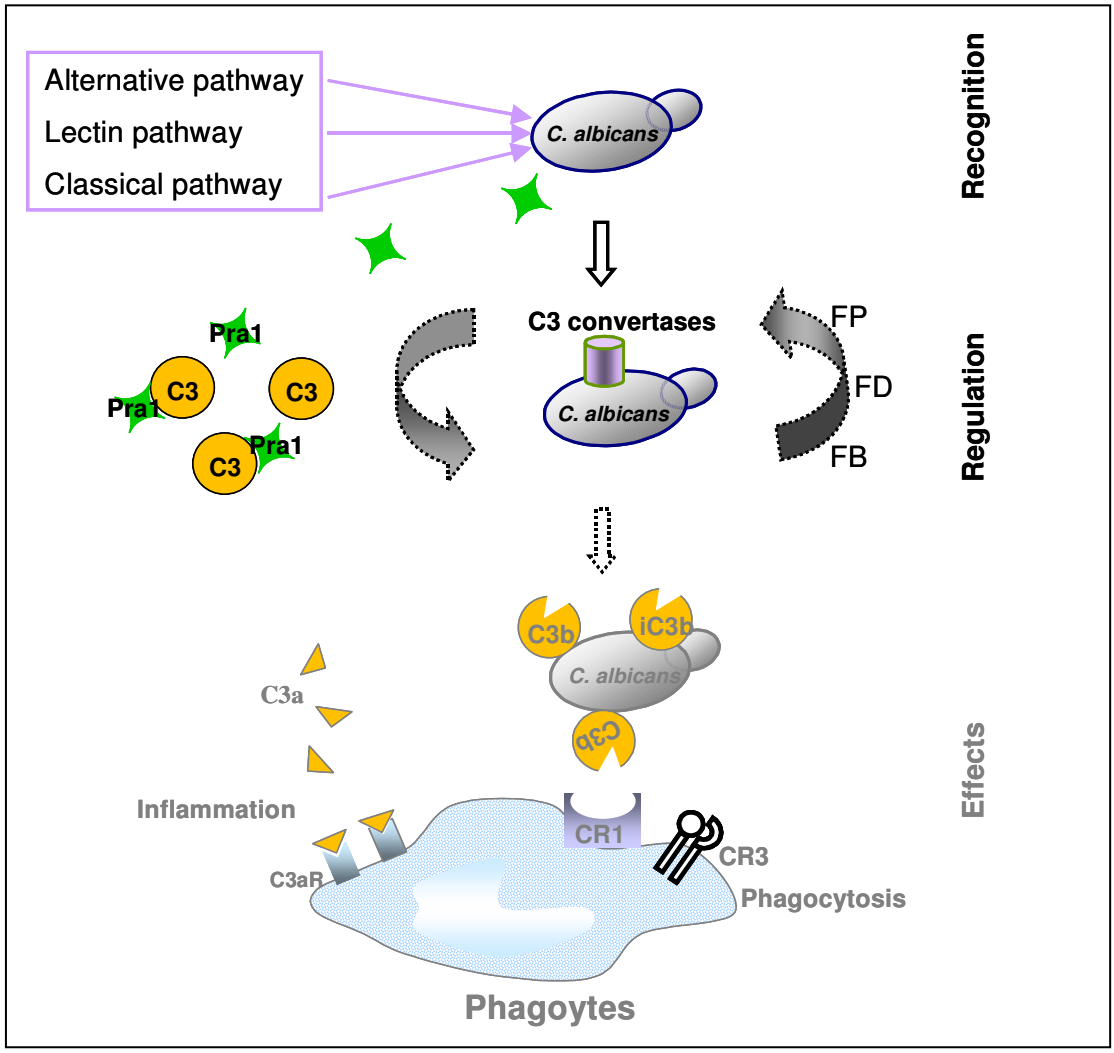

Figure 10. Secreted *Candida* Pra1 inhibits complement activation at level of C3. *C. albicans* secretes Pra1 into solution. Upon the complement activation, soluble Pra1 (i) complexes the central complement component C3, inhibits C3 activation and cleavage by C3 convertases, (ii) inhibits C3a release and C3b/iC3b deposition at *C. albicans* surface, and (iii) blocks the further complement progression and downstream complement effector functions, such as anaphylotoxin C3a and C5a based inflammation and C3b/iC3b mediated phagocytosis of the yeast by human macrophages.

Complement inhibitory effect of soluble Pra1 differs from Factor H binding

Soluble Pra1 is a potent complement inhibitor that blocks complement activation at the level of C3. Pra1 also binds Factor H. Factor H, as an AP complement regulator, controls the action of C3 convertase of the AP, prevents C3 convertase formation and also dissociates a preformed convertase [248]. Therefore, I defined whether Pra1 mediated blockade of complement activation is based on Factor H recruitment. It turns out that Pra1 blocks the lysis of sheep and rabbit erythrocytes, C3a release and also C3b/iC3b surface deposition also in ΔFactor H-HS (**manuscript 2**). In addition, the inhibitory effect of Pra1 on CP/LP activation further proves that the blockade of C3 conversion is independent of Factor H binding as Factor H only controls the AP but not the CP/LP. Therefore, this new function of Pra1 mediated complement inactivation and Factor H recruitment are independent from each other.

Function of Pra1 when bound to human cell surfaces

Pra1 binds to human cells

During the course of infection, *C. albicans* uses surface Pra1 to acquire Factor H, FHL-1 and C4BP as well as plasminogen to escape the first immune defense line, complement attack and to mediate the degradation of the extra-cellular matrices. However, facing the cellular response mainly mediated by human phagocytes (like neutrophils, macrophages and dendritic cells) and also non phagocytotic endothelial and epithelial cell barriers, *C. albicans* evolved ways to overcome it.

For the first time, I show that Pra1 expression level is up-regulated at fungal surface upon co-cultivation of *C. albicans* with human endothelial, epithelial cells and also monocytic cells (**manuscript 3**). This effect is consistent with the up-regulation of *PRA1* transcription when *C. albicans* adheres to epithelial cells [236]. This suggests that Pra1 plays a role in *C. albicans* interacting with human cells. Pra1, as a CR3 binding protein, binds to CR3 expressing human phagocytes, like U937 cells, THP-1 as well as neutrophils [235]. Interestingly, Pra1 also binds to non-phagocytic endothelial and epithelial cells that lack CR3 on the surface with even higher intensity (**manuscript 3**). Thus, these data indicate that the existence of a novel Pra1 receptor on the surface of human epithelial and endothelial cells. However, this new receptor is still unknown and needs to be further worked out.

Surface Pra1 binds to human endothelial cells and mediates C. albicans adhesion and invasion

Upon infection, *C. albicans* encounters different human cell types. Different cell types exert different effector functions against microbial invasion. For example, non-phagocytic endothelial and epithelial cells form physical barriers which *C. albicans* has to invade and overcome. Neutrophils or macrophages initiate a fast immune response upon infection, which *C. albicans* has to avoid or escape. Thus, pathogen and human cell interaction plays a complex and key role in the pathogenesis of microbial pathogens.

Upon co-culture of *C. albicans* with HUVEC cells, *C. albicans* either adheres or invades to the human endothelial cells (**manuscript 3**), which is considered as a critical step for the pathogenic yeast to overcome endothelial cell barrier and disseminate into deeper tissue layers [210]. Such cellular invasion of *C. albicans* has been demonstrated for porcine vascular explants [249], and also for brain microvascular endothelial cells [210,250,251,252]. Here, I show that surface attached Pra1 induces both adhesion and invasion of *C. albicans* into human endothelial cells that lack CR3. This interaction is blocked by specific Pra1 anti-serum, showing that Pra1 acts as a bridging molecule that mediates *C. albicans* contact with human endothelial cells (**Figure 11**). Similar to Pra1, two other surface proteins of *C. albicans*, Als3 and Hwp1 also induce fungal adhesion and invasion of host cells [182,253]. In addition, this feature seems conserved for many microbial pathogens, e.g. *S. pneumoniae, Toxoplasma gondii, Citrobacter freundii, Listeria monocytogenes* and *S. aureus* which also control and stimulate their own invasion of human endothelial cells via surface expressed virulence factors [254-258].

Secreted Pra1 binds to human phagocytes and blocks surface CR3 mediated recognition of human pathogens

Following invading and crossing a physical barrier, *C. albicans* must find ways to escape the recognition and surveillance by the human phagocytic cells. *C. albicans* secretes Pra1 which bind to CR3 at the surface of human phagocytic cells, and addition of Pra1 to *C. albicans* and neutrophil interaction system increases fungal survival [235] , which suggests that Pra1 may act as a decoy to block CR3 receptor, and inhibit further CR3 mediated-interaction with the surface expressed Pra1, thereby blocking the interaction or phagocytosis of yeast cells by human phagocytes (**Figure 11**).

In addition to protecting *C. albicans* from cell recognition, Pra1 also acts as a general inhibitor to block the recognition of human pathogens by human cells. The pneumococcal PspC protein recruits complement regulator Factor H to the cell surface of the human pathogenic *S. pneumoniae* via SCRs8-11 and SCRs19-20 of Factor H. In addition, Factor H binds CR3. Therefore, Factor H functions as a bridging protein and mediates *S. pneumoniae* entry into the CR3 expressing PMNs. This interaction progresses via a two-step mechanism. The initial contact is mediated through the interaction of pneumococcal-bound Factor H with glycosaminoglycans on the surface of human cells, while pneumococcal uptake depends on integrins (like CR3) and host signaling molecules such as PI3K. As CR3 is involved in such interaction, Pra1, as a CR3 binding protein efficiently inhibits Factor H mediated interaction of pneumococcus with human PMNs via binding to CR3. Furthermore, secreted Pra1 by binding to CR3 could also affect the cell signaling pathways (**manuscript 4**).

Taken together, Pra1 binds to phagocytic monocytes which express CR3 on the surface, and also binds to non-phagocytic human endothelial cells which lack CR3 on the surface via a novel receptor. Upon binding to human endothelial cells, surface expressed Pra1 mediates *C. albicans* adhesion and invasion to human endothelial cells. In addition, Pra1 acts an inhibitor that blocks the CR3 mediated recognition of pneumococcus by PMNs. Thus, secreted Pra1 may also act as a decoy to block CR3 on the surfaces of human phagocytes, thereby mediating the evasion of *C. albicans* from the cellular immune response. Consequently, *C. albicans* likely utilizes secreted Pra1 to form the third protective layer in the surrounding of the yeast cells, so that host phagocytes have no chance to come close to *C. albicans* (**Figure 11**). However, how *C. albicans* exactly controls and balances the usage of surface and soluble Pra1 for invading host epithelial and endothelial cell barriers and escaping the host phagocytic cells is still a question.

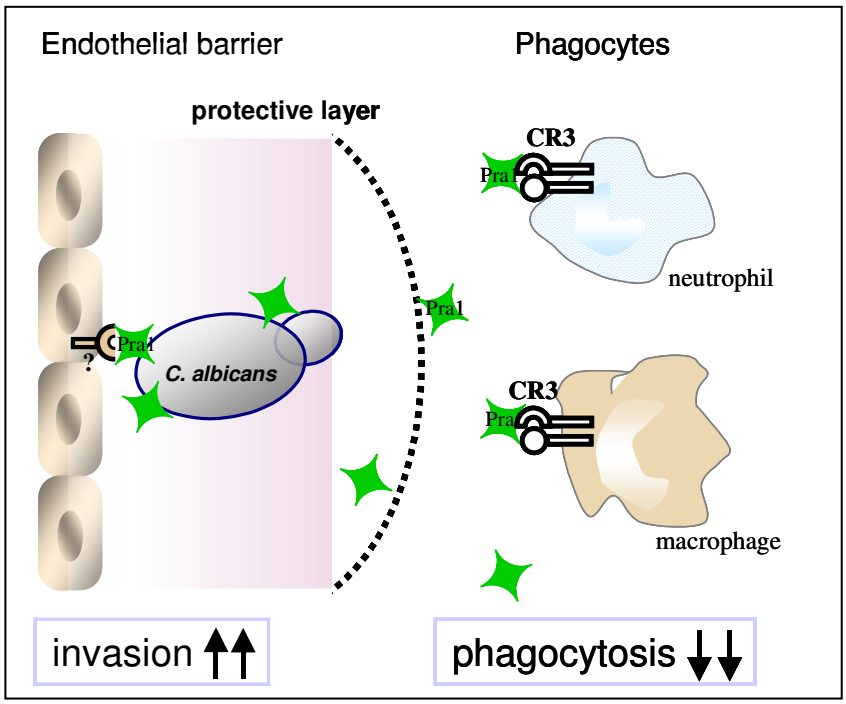

Figure 11. Pra1 interferes with *C. albicans* interaction with human cells. Surface Pra1 binds to human non-phagocytic, CR3 deficient endothelial cells via a novel unknown receptor. Upon binding, Pra1 mediates *C. albicans* adhesion and invasion to human endothelial cells. In addition, Pra1 acts an inhibitor that blocks the CR3 mediated recognition of pneumococcus by PMNs. Thus, soluble Pra1, via binding to CR3, may act as a decoy, block CR3 mediated interaction of phagocytes with *C. albicans*, thereby blocking further CR3 mediated immune responses.

Surface expression and sequence variation of *PRA1* in clinical *C. albicans* isolates

Pra1 elicits a strong immune response in infected patients [259]. In this work, Pra1 was defined as a multiple functional virulence factor which plays a key role in mediating *C. albicans* for an infection. In order to correlate the *in-vitro* test of recombinant Pra1 with *in-vivo* situation of the native Pra1, I compared Pra1 surface expression levels and sequence variation in clinical isolates derived from different infected patients to that in wild type *C. albicans* strain. Pra1 is expressed at the surface of all tested clinical isolates. Pra1 surface expression levels in clinical isolates are either higher than or comparable to that of the wild type SC5314 strain. Sequence analysis of the *PRA1* gene from each clinical isolate shows that Pra1 is a relatively conserved gene and is not as polymorphic as other Factor H/C4BP binding proteins of pathogens, including CRASPs of *Borrelia* species [260] or M protein from *S. pyogenes* [261]. In the 897-base-pair-long protein coding region, sixteen nucleotides are changed in the tested clinical isolates. These exchanges, which occur either in homozygous or in heterozygous scenarios were observed with different frequencies. Most of these changes do not affect the protein sequence or only cause the conservative changes of amino acids, such as Asp90Glu, Glu101Asp,

Ser154Thr, as well as Asp225Glu. However, three modulations in the 5'-terminus of the *PRA1* affect the protein sequence. For example, both a polar, uncharged Asn- at position 25 and a non-polar Gly residue at position 105 are changed to negatively charged Asp residues. In addition, a non polar Ile residue at position 111 is changed to a polar, uncharged Ser residue. Interestingly, A73G exchange that affects the Pra1 protein sequence (Asn25Asp) is observed in all tested clinical isolates with the same homozygousity, in comparison with the standard Pra1 derived from the wild type strain SC5314 (**manuscript 3**). This exchange likely represents the most relevant site of the *PRA1* gene for an infection.

Conclusions-Pra1 is a multifunctional virulence factor which favors fungal survival at different sites

Pra1 is a surface protein and also released by both *Candida* yeast cells and hyphae, and does also bind to human cells [235,262]. The different locations allow *Candida* Pra1 to display different functions at distinct sites: (i) ***as a surface protein***, Pra1 binds and acquires human complement regulators Factor H, FHL-1 and C4BP as well as plasminogen to the surface of the *C. albicans*. The attached human regulators display functional activities, control complement attack and mediate degradation of extra cellular matrixes, and consequently aid in *Candida* infection (**manuscript 1**); (ii) ***as a secreted protein***, Pra1 complexes C3 in solution, blocks C3 cleavage by C3 convertases of both the AP and the CP/LP, inhibits further complement amplification and progression. Consequently Pra1 displays an anti-inflammatory effect by inhibiting the release of anaphylatoxins C3a and C5a and reduces attachment and uptake of yeast cells by human macrophages by blocking C3b/iC3b surface deposition (**manuscript 2**). Pra1, via binding Factor H, also enhances complement control mediated by complement regulator Factor H in fluid phase. In addition, secreted Pra1 binds back to the surface of *C. albicans* and functions as a surface protein; (iii) ***binding to the surface of host cells***. Upon co-cultivation of *C. albicans* with human cells, Pra1 surface expression is highly up-regulated. Surface Pra1 by binding to human non-phagocytic, CR3 deficient endothelial via a novel receptor mediates *C. albicans* adhesion and invasion into the human endothelial cells (**manuscript 3**). In addition, secreted Pra1, via binding to surface integrin CR3, efficiently inhibits Factor H mediated interaction of pneumococcus with human epithelial and PMNs (**manuscript 4**) (**Figure 12**). Homozygous sequence variation of Asn25Asp likely represents a highly relevant site of the Pra1 for an infection.

In summary, Pra1, as a multiple functional fungal complement inhibitor, apparently forms multiple protective layers which efficiently shield the human pathogenic yeast *C. albicans* from the humoral and cellular host immune responses. In addition, surface Pra1 acts as a bridging molecule to mediate *C. albicans* adhesion and invasion to non-phagocytic cell barriers. The multiple functions of *Candida* Pra1 provide an example for the multiplicity and complexity of the immune escape that are contributed by one single fungal virulence factor. A detailed understanding of these multiple roles of Pra1 allows to define new strategies to interfere with and fight against *C. albicans* infection.

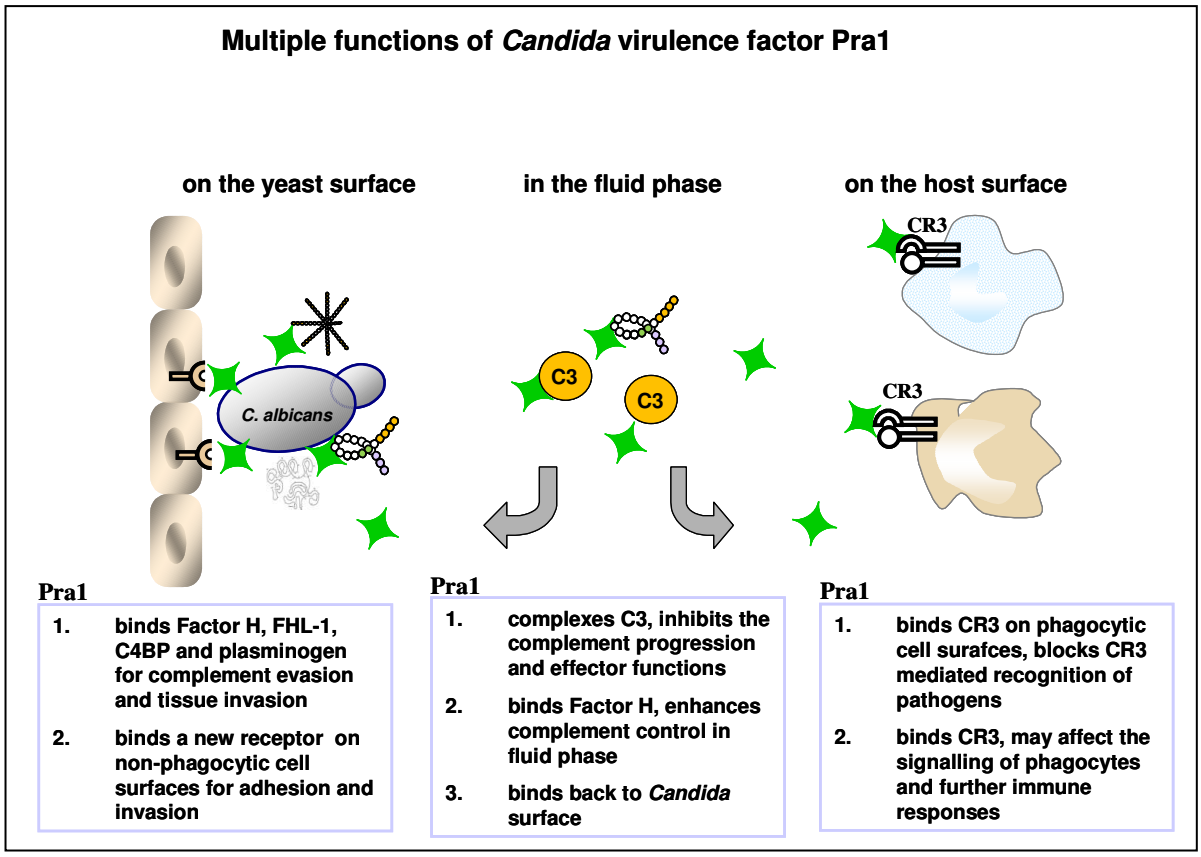

Figure 12. The multiple functions of *Candida* Pra1. As a surface protein, Pra1 acquires Factor H, FHL-1, C4BP as well as plasminogen to the surface of *C. albicans* to mediate the complement evasion and degradation of extra-cellular matrices. Surface Pra1 can also act as a bridging molecule to mediate *C. albicans* adhesion and invasion to human non-phagocytes via binding to a novel receptor. As a secreted protein, Pra1, by complexing C3 in solution, blocks C3 conversion, thereby inhibiting further complement progression and the effector functions. Secreted Pra1 binds to Factor H and enhances the complement control of Factor H in fluid phase. In addition, secreted Pra1 binds back to *C. albicans* surface and functions as a surface protein. When binding to the CR3 on the phagocyte surfaces, Pra1 blocks further recognition of pathogens by phagocytes via CR3, and may also affect the signaling modulation of the phagocytes.

6. Summary

C. albicans is an opportunistic human pathogen that causes superficial, as well as life-threatening infections in immuno-compromised patients. In order to establish an infection, *C. albicans* has developed multiple mechanisms to avoid host immune recognition and cross host tissue barriers. The aim of the work was to identify proteins from *C. albicans* that mediate immune, particularly complement evasion and to characterize these proteins on a molecular level. In this project, I identified and defined *C. albicans* Pra1 as a multifunctional virulence factor which mediates *C. albicans* for immune evasion and tissue invasion, thereby favoring fungal infection. Using the polyclonal Pra1 antis-serum, Pra1 was localized as a yeast surface protein and also secreted into the culture medium. Different locations specialize Pra1 different functions, (i) ***as a surface protein***, Pra1 binds human complement regulators Factor H, FHL-1 as well as plasminogen. In addition, Pra1 is the first fungal C4BP binding protein. The attached human regulators mediate complement evasion and degradation of extra-cellular matrices, and consequently favoring *C. albicans* invasion; (ii) ***as a secreted protein***, Pra1 was identified as the first fungal complement inhibitor. Pra1 complexes C3 in solution, blocks C3 cleavage by C3 convertases, thereby inhibiting further complement amplification and progression, and downstream complement effector functions, such as the inflammatory anaphylatoxins C3a and C5a generation, C3b/iC3b surface opsonization, as well as C3b/iC3b mediated adhesion and uptake of the yeast by human macrophages; and (iii) ***binding to the surface of human cells***. Upon co-culture of *C. albicans* with human cells, Pra1 surface expression is up-regulated. For the first time, I show that Pra1 binds to human endothelial and epithelial cells in a CR3 independent manner via a novel receptor. Upon binding, surface Pra1 functions as an invasin and mediates *C. albicans* for adhesion and invasion into human endothelial cells, thereby likely allowing this fungal pathogen to cross the tissue layers and cause invasive disease. In addition, Pra1 acts as an inhibitor that binds to CR3 and blocks CR3 mediated recognition of pneumococcus by PMNs.

In addition, I -for the first time-, identified Pra1 surface expression and sequence variation in clinical isolates derived from different infected patients. Pra1 is expressed at the surface of all tested clinical isolates. All tested isolates shows either higher or comparable Pra1 surface levels, as compared to the wild type *C. albicans* strain SC5314. Sequence analyses of the *PRA1* gene identify a relevant nucleotide exchange (A73G) which also affects the amino acid (Asn25Asp) in all test isolates with homozygousity. Thus, Pra1 is a multifunctional virulence

factor that favors fungal survival at distinct sites. A detailed understanding of these multiple roles of Pra1 may allow to defining new strategies to interfere with and fight against *C. albicans* infection.

Zusammenfassung

Der Mensch verfügt und nutzt ein komplexes und streng reguliertes Immunsystem das den Organismus vor infektiösen Erregern schützt. Allerdings haben pathogene Erreger im Lauf der Evolution gelernt die verschiedenen Immunbarrieren zu überwinden, sich in einem immunkompetenten Wirt auszubreiten und so Krankheiten zu verursachen. *Candida albicans* ist ein opportunistischer humanpathogener Erreger, der sowohl Oberfächeninfektionen aber auch systemische Erkrankungen verursachen kann. *C. albicans* verfügt über ein breites Arsenal von Mechanismen und Immunevasionsstrategien mit denen es dem Erreger gelingt die Immunabwehr des Menschen als dessen Wirt zu inhibieren. Ziel dieser Arbeit war es Virulenzfaktoren der humanpathogenen Hefe *C. albicans* zu identifizieren und diese zu charakterisieren. Dabei wurde Pra1 Protein (pH regulated antigen 1) der Hefe als multifunktioneller Virulenzfaktor identifiziert. Pra 1 steuerte die Immunevasion auf mehreren Ebenen, Pra1 agiert (i) als Komplement Inhibitor, (ii) als Faktor H, FHL1 und Plasminogen bindendes Protein, (iii) Pra1 bindet auch den Regulator des klassischen Weges C4BP und (iv) Pra 1 bindet an mindestens zwei Rezeptoren auf der Oberfläche von humane Makrophagen und Endothelzellen. Pra1 führt diese vielfältigen Funktionen in drei unterschiedlichen Kompartimenten aus: als **Oberflächenprotein** bindet Pra1 die Komplement Regulator Faktor H, FHL1, C4BP sowie Plasminogen und verhindert so die Opsonisierung der Oberfläche mit C3b bzw. erlaubt die Degradation von Extrazellulären Matrixproteinen. Als **sezerniertes Protein** wurde Candida Pra1 als erste Komplementinhibitor identifiziert, der freies C3 bindet und so die weitere Aktivierung dieser zentralen Komplementkomponente verhindert. So kann die Hefe die Aktivierung des Komplement Kaskade, die Bildung von toxischen Aktivierungsprodukten sowie die Opsonierung und Phagozytose effizient blockieren. A**uf der Oberfläche** von human Zellen bindet Candida Pra1 an spezifische Rezeptoren. In dieser Arbeit wurde ein neuer Pra1 Rezeptor der auf humanen Endothel- und Epithelzellen exprimiert wird identifiziert. Pra1 ist ein wichtiger Virulenzfaktor der humanpathogenen Hefe *Candida albicans*, der vielfältigen Schritte der Immunevasion der humanpathogene Hefe kontrolliert und steuert und der an unterschiedlichen Kompartimenten agiert. Eine Inaktivierung dieses zentralen Virulenzfaktors bietet eine Möglichkeit die Infektionen mit der humanpathogenen Hefe im menschlichen Organismus effizient zu kontrollieren.

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8. Eigenständigkeitserklärung

- Die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena ist mir bekannt.
- Die vorliegende Dissertation wurde von mir selbst angefertigt und alle benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen sind in dieser Arbeit angegeben.
- Alle Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben, habe ich benannt.
- Die Hilfe eines Promotionsberaters habe ich nicht in Anspruch genommen.
- Dritte Personen haben weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.
- Diese Arbeit wurde bisher weder an einer anderen Hochschule als Dissertation noch als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht.

Jena, 14. 04. 2010

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Shanshan Luo

9. Overview of publication list, oral, poster presentations and award

Publication list

1. **Shanshan Luo**, Sophia Poltermann, Anja Kunert, Steffen Rupp, Peter F. Zipfel. Immune evasion of human pathogenic yeast *Candida albicans*: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein. *Mol Immunol*. 2009 Dec; 47(2-3):541-50.
2. Peter F. Zipfel, **Shanshan Luo**, Susann Schindler. Immunbiologie von Humanpathogenen Hefen und Schimmelpilzen. Wohnmedizin 2009.
3. **Shanshan Luo,** Andrea Hartmann, Hans-Martin Dahse, Christine Skerka, Peter F. Zipfel. Secreted pH-regulated antigen 1 of *Candida albicans* blocks activation and conversion of complement C3. *J Immunol*. Jul 19th, 2010 (in press).
4. **Shanshan Luo,** Anna M. Blom, Steffen Rupp, Bernhard Hube, Uta-Christina Hipler, Christine Skerka, Peter F. Zipfel. The pH-regulated antigen 1 of *Candida albicans* interacts with C4b-binding protein (C4BP) and mediates fungal contact with human endothelial cells. (in revision at the Journal of Biological Chemistry, 2010)**.**
5. Vaibhav Agarwal, Tauseef M. Asmat, **Shanshan Luo,** Inga Jensch, Peter F. Zipfel, and Sven Hammerschmidt. Complement regulator Factor H mediates a two-step uptake of *Streptococcus pneumoniae* by human cells. *J Biol Chem.* 2010 Jul 23; 285(30):23486-95.
6. Teresia Hallström*, **Shanshan Luo***, Corinna Siegel, Matthias Mörgelin, Sven Hammerschmidt, Kristian Riesbeck, Peter Kraiczy, Christine Skerka, and Peter F. Zipfel. Inhibition and control of the terminal complement pathway is a common immune escape strategy used by diverse pathogens. (*Both authors contributed equally to this work) (In preparation).

Oral presentations

1. **Shanshan Luo**, Andrea Hartmann, Hans-Martin Dahse, Christine Skerka, Peter F. Zipfel. CRASP2 of the human pathogenic yeast *Candida albicans* is a complement inhibitor that blocks C3 conversion. The XXIII International Complement Workshop, August 1st-5th, 2010, New York, NY, USA;
2. Danny Kupa, **Shanshan Luo**, Peter F. Zipfel. *Candida albicans* pH-regulated antigen 1 is a potent fungal protein for complement evasion 3rd Joint Conference German Society for Hygiene and Microbiology (DGHM) and Association for General and Applied Microbiology; March 28th-31st, 2010; Hannover, Germanny;
3. **Shanshan Luo** and Peter F. Zipfel. Immune escape of *Candida albicans*: many faces of

Pra1. DFG Priority meeting SPP 1160, October 15th-16th, 2009, Jena, Germany;

4. **Shanshan Luo** and Peter F. Zipfel. Immune escape of *Candida albicans*: many faces of CRASP2. 3rd Joint of ILRS and JSMC symposium, October 12th-13th, 2009, Jena, Germany;
5. **Shanshan Luo** and Peter F. Zipfel. Immune escape of *Candida albicans*: the many faces of Pra1. HKI colloquium, February 24th, 2009, Jena Germany;
6. **Shanshan Luo** and Peter F. Zipfel. Characterization of CRASP-2, a novel surface protein of *Candida albicans* which mediates immune evasion and adhesion to host cells. 2nd ILRS Symposium, September 15th-16th , 2008, Dornburg, Germany (Sep 2008);
7. **Shanshan Luo**, Uta-Christina Hipler, Peter F. Zipfel. Characterization of CRASP-2, a novel surface protein of *Candida albicans* which mediates immune evasion and adhesion to host cells. DMycG conference, September 4th-6th, 2008, Jena, Germany;
8. **Shanshan Luo** and Peter F. Zipfel. Characterization of *Candida albicans* proteins that interacts with human complement regulators. 1st ILRS Symposium, September 16th-17th , 2007, Dornburg, Germany;
9. **Shanshan Luo**, Anja Kunert, Peter F. Zipfel. Characterization of *Candida albicans* proteins that interacts with human complement regulators. Status workshop of the DGHM-Fachgruppe "Eukaryotic pathogens", February 23rd-24th, 2007, Stuttgart, Germany.

Poster presentations

1. **Shanshan Luo**, Anna M. Blom, Steffen Rupp, Bernhard Hube, Uta-Christina Hipler, Christine Skerka, Peter F. Zipfel. CRASP2 of *Candida albicans* binds C4b-binding protein and mediates fungal contact with human endothelial cells. The XXIII International Complement Workshop, August 1st-5th, 2010, New York, NY, USA;
2. **Shanshan Luo**, Andrea Hartmann, Hans-Martin Dahse, Christine Skerka, Peter F. Zipfel. CRASP2 of the Human Pathogenic Yeast *Candida albicans* is a complement inhibitor that blocks C3 conversion. *Mol Immunol*, 2009; 46 (14); 2857. The XII European Complement Workshop, September 5th-9th, 2009, Visegrad, Hungary;
3. **Shanshan Luo**, Sophia Poltermann, Anna Blom, Peter F. Zipfel. Characterization of CaCRASP-2, a novel surface protein of *Candida albicans* involved in immune evasion and infection to host cells. *Mol Immunol*, 2008; 45 (16); 4170. The XXII International Complement Workshop, September 28th - October 2nd 2008, Basel, Switzerland;
4. **Shanshan Luo**, Sophia Poltermann, Uta-Christina Hipler, Peter F. Zipfel. Immune

evasion of *Candida albicans*: identification and characterization of Factor H binding proteins. *Mol Immunol*, 2008; 45 (16); 4171. The XXII International Complement Workshop, September 28th - October 2nd 2008, Basel, Switzerland.

Awards

1. Travel award for the XXII International Complement Workshop, September 28th – October 2nd, 2008, Basel, Switzerland.
2. Travel award for the XXIII International Complement Workshop, August 1st –5th, 2010, New York, USA.

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